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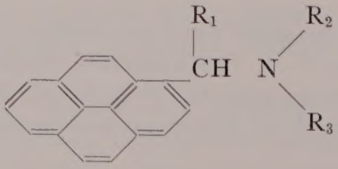
ERRATA

Vol. 21, page 2, line 8, "propably" should read "probably"
Vol. 21, page 5, line 16, "mount" should read "amount"
Vol. 21, page 11, footnote[2], "Tompson" should read "Thompson"
Vol. 21, page 24, Table I, structure should be:



Vol. 21, page 81, line 1, "pines" should read "pins"
Vol. 21, page 85, line 8, "good" should read "wood"
Vol. 21, page 93, line 2, "varies" should read "varied"
Vol. 21, page 125, Table VII, column 2, lines 11, 12, 13 should read

"15.0 g./l."
0.10 "
0.50 "

Vol. 21, page 175, footnote[1] should read " . . . under a grant (No. G17524) by . . . "

Vol. 21, page 201, line 2 from bottom, "2431" should read "2413"

Vol. 21, page 228, line 21, "Fig. 5" should read "Fig. 3"

Vol. 21, page 250, line 13 from bottom, "competed" should read "completed"

Vol. 21, page 319, line 12, "Spotlight" should read "Stoplight"

Vol. 21, page 323, line 9 from bottom, "7" should read "10"

Vol. 21, page 352, line 8 from bottom, "uilization" should read "utilization"

Vol. 21, page 364, top line should read "Chambers 1, 3, 5, and 6 contained 0.05 M H_3BO_3 adjusted to pH 8.0 with NaOH."

Vol. 21, page 367, next to last sentence of legend for Figure 5 should read "All chambers contain 0.05 M H_3BO_3 neutralized to pH 8.0 with NaOH. Chambers 2, 4, 6, and 7 contained in addition NaCl at the indicated molarities."

Vol. 21, page 387, line 3 of title, "DIFERENT" should read "DIFFERENT"

Vol. 21, page 387, line 12 in SUMMARY, "was" should read "were"

Vol. 21, page 390, Table II, line 2 of heading, a period should follow "in Nutrient Solutions"

Vol. 21, page 449, Figure 1, insert line directly below A to divide Figure 1 into two parts.

INDOLE-3-ACETYL-D,L-ASPARTIC ACID AS A NATURALLY-OCCURRING INDOLE COMPOUND IN TOMATO SEEDLINGS

VAS V. ROW,[1] WILLIAM W. SANFORD,[2] AND A. E. HITCHCOCK

SUMMARY

An Ehrlich-positive substance chromatographically identical with synthetic indole-3-acetyl-D,L-aspartic acid (IAAP) was present in ethanolic extracts of control seedling tomato plants 30 days old. There was no detectable indole-3-acetic acid (IAA) in the same extract. Extracts of tomato plants pretreated with IAA or 1-naphthaleneacetic acid contained IAA and considerably more IAAP than extracts of control plants. This is believed to be the first report of naturally-occurring IAAP in plants. Andreae and co-workers detected IAAP in plants pretreated with IAA but not in controls of 12 species including tomato. The results lead to the suggestion that the possible role of IAAP in normal metabolism should be investigated, especially in plants which have different photoinductive requirements for flowering.

INTRODUCTION

In 1955 Andreae and Good (1) presented evidence of indole-3-acetyl-D,L-aspartic acid (IAAP) in etiolated pea tissue incubated with indole-3-acetic acid (IAA) but not in control pea tissue. Similar results were obtained later (4) with 12 species of plants including pea and tomato. The results were interpreted as evidence for a detoxication mechanism in plants for handling excess amounts of exogenously applied IAA. Even through their results did not support the concept that endogenous IAA is conjugated with aspartic acid, this possibility was not precluded (4).

Results are presented which show that extracts of control tomato (*Lycopersicon esculentum* Mill.) plants (30 to 35 days old from seed) contain a stable substance that is chromatographically identical with synthetic IAAP.[3] It now seems likely that the stable Ehrlich-positive substance extracted from tomato and tentatively referred to as indole-3-pyruvic acid (5) was, in fact, IAAP.

The availability of synthetic samples of both compounds has made their identification in tissue extracts more certain. Synthetic samples of indole-3-pyruvic acid (IPyA) are reported to be unstable in storage and during chromatography on paper, especially in solvents containing ammonia, and produce one or more decomposition products (2, 8). Chromatograms of

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[3] Supplied through the courtesy of Dr. Norman E. Good, Science Service Laboratory, London, Ontario, Canada.

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IPyA sprayed with Ehrlich's reagent develop a yellow-green color (2). In contrast, IAAP is stable and yields a purple product with Ehrlich's reagent.

In addition to the results showing that IAAP was present in extracts of tomato, there is reason for believing that the compound extracted from soybeans (*Glycine max* Merr.) and tobacco (*Nicotiana tabacum* L. var. Maryland Mammoth) leaves, and tentatively referred to by Vlitos and Meudt (11) as IPyA, was propably IAAP. Special interest centers on this possibility since measurable quantities of the compound were found in extracts of Biloxi and Lincoln soybeans and leaves of Maryland Mammoth tobacco grown under photoinductive cycles (8 hrs.) but little or none in extracts of similar plants grown under long-day conditions (18 hrs.). Although the compound in extracts had the same Rf (0.21) as a synthetic sample of IPyA, the former was stable and the latter highly unstable (11). No mention was made of the color of the "heavily-colored spots at Rf 0.21." However, in a recent report Vlitos (9) stated that the stable compound extracted from soybeans and tobacco (11) could not be IPyA.

It seems evident from the results thus far mentioned that the substance extracted from soybeans (11) was not IPyA. Further work would be required in order to determine whether Biloxi and Lincoln soybeans grown under eight-hour photoperiods do contain IAAP. If this is found to be true, the possible role of IAAP in normal metabolism should be investigated, especially in species which have different photoinductive requirements for flowering.

The present report describes the comparative results of chromatography, on paper, of a synthetic sample of IAAP and IAAP extracted from control tomato plants and from tomato plants pretreated with IAA or 1-naphthaleneacetic acid (NAA). Certain unpublished data obtained before the synthetic sample of IAAP was available are also included.

MATERIAL AND METHODS

Tomato plants 30 to 35 days old were used in all the tests. The plants were harvested for extraction 24 hours after treatment, by which time characteristic epinastic responses were observed. Extraction and chromatography of the plant material were carried out by a modification of methods used by Kefford (6) and Vlitos and Meudt (10).

Extraction. The plant material was quick-frozen in a mixture of solid carbon dioxide and methanol for two hours. The frozen material was ground in a prechilled Waring Blendor at −7° C. Aliquots of 150 to 200 g. of the tissue homogenate were extracted with 500 ml. absolute ethanol at −7° C. for 20 hours. The extract was filtered through Whatman No. 1 paper into a vacuum flask washing the residue repeatedly with absolute ethanol until 1,000 ml. of the extract was collected. The ethanolic extract was then evaporated under reduced pressure at room temperature until only the aqueous fraction was left. The aqueous fraction was filtered, the residue washed with saturated sodium bicarbonate solution, and the washings combined with the filtrate. The filtrate mixture was then adjusted to pH 9 with 1*N* NaOH and shaken for 3 minutes with a half volume of peroxide-free ethyl ether in a separatory funnel. At the end of an hour the the alkaline-ether fraction was separated from the aqueous fraction and discarded. Two such ether extractions were carried out. The aqueous fraction was then adjusted to pH 3 with 1*N* HCl and extracted three times with half volumes of ether keeping an interval of one hour between extractions. The ether extracts were combined and evaporated in the hood. The residue was taken up in a small volume of isopropanol so that a 10-μl. aliquot of the extract represented 2 g. of plant tissue.

Chromatography. Indole compounds were separated by ascending paper chromatography on Whatman No. 1 paper. A volume of extract corresponding to between 6 and 24 g. of tissue was spotted.

Quantitative determination of IAA and IAAP. Known amounts of synthetic IAA were chromatographed on paper and the density of the spots used to construct a calibration curve (Fig. 1). This curve was used to calculate the amount of IAA in chromatographed extracts of IAA-treated tomato plants. The extract eluted from undeveloped paper chromatograms at the Rf of the unknown compound (IAAP) was concentrated and assigned a value of one unit. This extract and dilutions of it were chromatographed and the density of the spots used to construct a calibration curve (Fig. 1) which was used in other tests to determine the amount (units) of IAAP in extracts of tomato tissue.

RESULTS WITH SYNTHETIC AND EXTRACTED IAAP

After separation on paper, synthetic samples of IAA and IAAP yielded purple spots with Ehrlich's reagent at Rf 0.65 and Rf 0.30, respectively (Fig. 2 A). Extracts of control tomato plants yielded spots at an Rf value corresponding to synthetic IAAP (Fig. 2 B), but no IAA was detected (<0.2 μg./24 g. tissue). Extracts of tomato plants treated with IAA gave spots at an Rf value corresponding to synthetic IAA, representing part of the added IAA recovered in the extract. A second spot appeared at the same Rf as synthetic IAAP (Fig. 2 C). Extracts of tomato plants treated with NAA also gave spots at Rf values corresponding to synthetic IAA and synthetic IAAP (Fig. 2 D).

Additional characterization of extracted IAA and IAAP was obtained by co-chromatography with synthetic compounds and by two-dimensional chromatography of the extracts. In both cases single spots appeared at the Rf values corresponding to those shown in Figure 1 for IAA and IAAP.

Eluates from nonsprayed chromatograms of extracts of IAA-treated

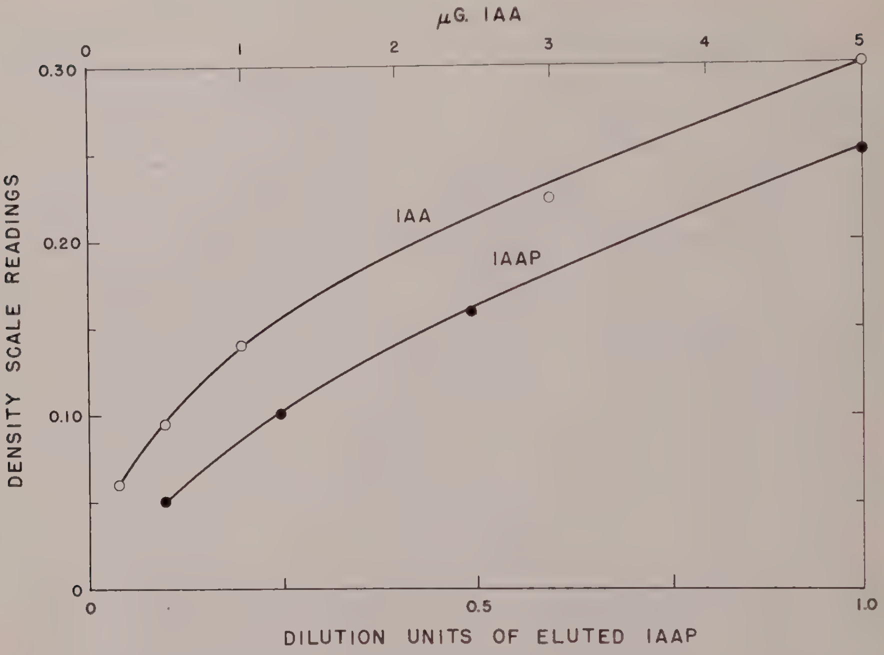

FIGURE 1. Proportionality between synthetic IAA and IAAP eluted from chromatograms of tomato extract. The extract of IAA-treated tomato plants eluted from undeveloped chromatograms at the Rf position for IAAP was concentrated and assigned a value of one unit. This concentrated extract and dilutions of it were chromatographed and the density of the spots compared with spots of synthetic IAA. The calibration curve for IAAP was used for the determination of IAAP units in other tests.

tomato plants were active in the slit pea (*Pisum sativum* L.) test. Eluted IAAP was considerably less active than eluted IAA, but the tests were not carried out on a quantitative basis.

EXTRACTION FROM TOMATO OF A COMPOUND SIMILAR TO IAAP

In some earlier experiments paper chromatography was used to separate and detect IAA in ethanolic extracts of tomato plants pretreated with IAA and other growth regulants. An unknown compound giving a purple color on chromatograms sprayed with Ehrlich's reagent was detected consistently in extracts of control and treated tomato plants. The Rf value of this compound was about 46 per cent of that for synthetic IAA. The color reaction and Rf were similar to those of synthetic IAAP (Fig. 2). Assuming the compound to be IAAP, some of the earlier results reported previously in abstract form (5) appear to warrant a more detailed description. Subsequently, in the present report the compound will be referred to as IAAP.

After injecting IAA into tomato plants the amount of extractable IAAP increased to a maximum and IAA decreased to a low level in 48 hours (Fig. 3 and Table I). In tests of longer duration IAAP decreased until it reached the level in control plants at the end of 96 hours. Trace amounts of IAA were detected after 72 hours but none at the end of 96 hours (Fig. 3). The amount of IAAP also increased with the amount of injected IAA (Fig. 4). Perhaps much of the injected IAA conjugated with aspartic acid to form IAAP as postulated by Andreae and Good (1). However, this explanation does not account for the substantial increase in IAAP in extracts of plants treated with NAA and ethylene. The amount of IAAP extracted from 2,4-D-treated plants was the same or slightly more than was extracted from control plants (Table I).

When the amount of IAA added to ground frozen tomato tissue was less than 1 μg. per gram harvested tissue, only 50 per cent of the IAA could be recovered. The addition of 10 μg. IAA per gram of harvested tissue resulted in more than 90 per cent recovery. However, the mount of IAA extracted from IAA-treated plants was proportional to the amount of treated tissue. Likewise, the amount of extractable IAAP was proportional

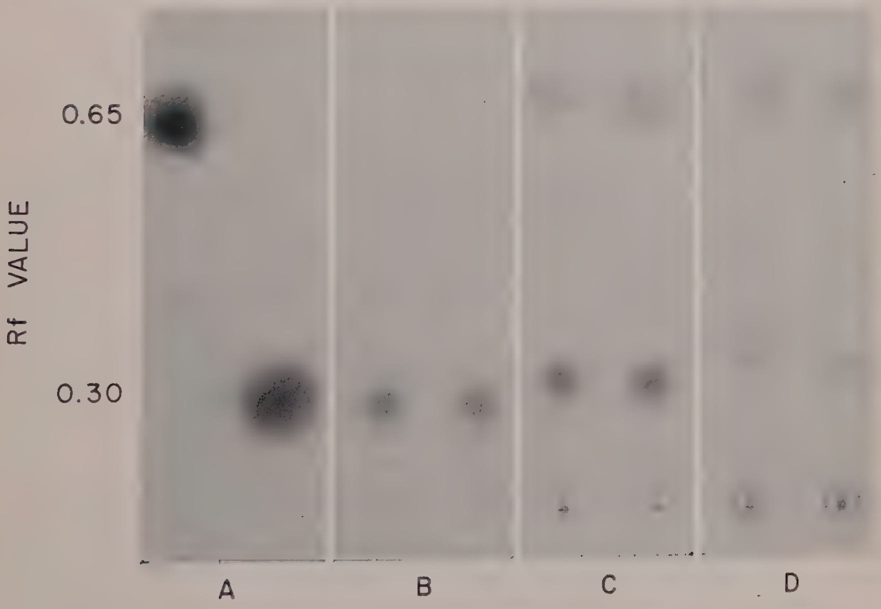

Figure 2. Paper chromatograms of extracts of control and treated tomato plants compared with synthetic samples of IAA and IAAP developed in isopropanol—ammonia—water (80:5:15 v/v) solvent and sprayed with Ehrlich's reagent. (A) Ten μg. each IAA, Rf 0.65 and IAAP, Rf 0.30; (B) two aliquots of control tomato plant extract each representing 24 g. tissue, Rf 0.30; (C) two aliquots of extract of IAA-treated tomato plants each representing 6 g. tissue (Rf 0.65 and Rf 0.30); (D) same as C, but for NAA-treated plants.

TABLE I

AMOUNT OF IAAP EXTRACTED FROM TOMATO PLANTS PRETREATED WITH GROWTH REGULATORS

Compound	Amount injected, µg./g. tissue	Duration of treatment, hrs.	Epinasty induced*	Extracted IAAP,** % of control
IAA	3 –5.5	2– 3 18–24 42–49	++++ ++++ +	125–150 150–200 200–250
NAA	1.5–2.5	2– 3 18–24 42–49	+ ++++ ++++	125–150 125–150 150–200
2,4-D	0.7–1.8	2– 3 18–24 42–49	0 +++ ++++	100–125 100–125 100–125
Ethylene	†	18–24	++++	200–250

* 0 = None; + = slight increasing to ++++, pronounced.
** Not identified, but having same characteristics as shown in Figure 1.
† Plants exposed in closed chamber to 10 p.p.m. ethylene.

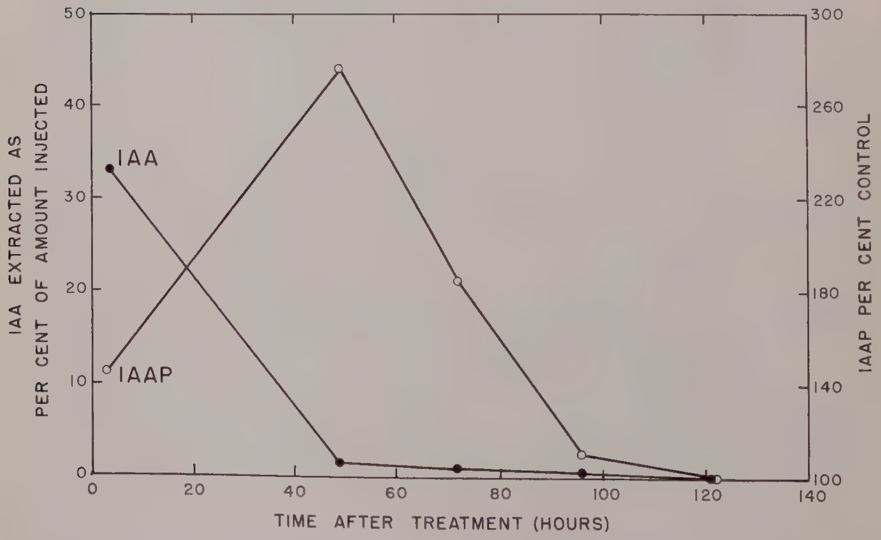

FIGURE 3. Change in amount of IAA and IAAP extracted from tomato plants after an injection treatment with IAA equivalent to 6.7 µg. per g. tissue.

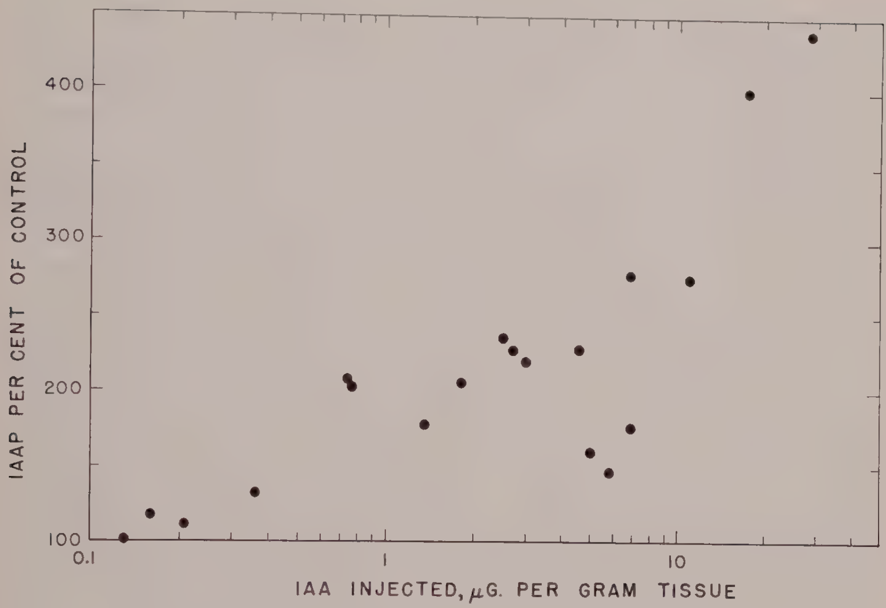

Figure 4. Relation between amount of IAA injected into tomato plants and the amount of IAAP extracted.

to the amount of control and IAA-treated tomato plant tissue, and more IAAP was extracted from control tomato plants in summer than in winter (Fig. 5). The concentration of eluted IAAP in a dilution series was proportional to the concentration of standard solutions of IAA (Fig. 1). It was further shown that a linear relationship exists between the fluorescence of the unidentified substance (now presumed to be IAAP) and the amount of tissue represented by chromatographic spots located at Rf 0.25 to 0.29 by means of an ultraviolet lamp (7). IAA located at Rf 0.54 on the same chromatogram showed a similar proportionality between the amount per spot and fluorescence.

DISCUSSION

The present results showing detectable amounts of IAAP in extracts of control tomato tissue are in contrast to the results of Good *et al.* (4) which showed no detectable IAAP in the control tissue of 12 species of plants. However, these workers did not preclude the possibility that endogenous IAA conjugated with aspartic acid in normal plant metabolism, even though their results did not lend support to this concept. Failure to detect IAAP in control plant tissue (4) may have been due to differences in extraction and chromatographic procedures as compared with those used in the present tests. For example, in the present tests the chromatographed extracts represented 6 to 24 g. tissue per spot as compared with 3 g. tissue used by Good *et al.* (4). Correspondingly, in the present tests the frozen ground tissue was extracted with ethanol at −7° C. as compared with the addition of sodium bicarbonate solution to the frozen tissue before grinding.

Tomato plants pretreated with IAA, NAA, or ethylene yielded extracts containing one and one-half to two times as much of the unidentified substance assumed to be IAAP as was extracted from control plants. These results show that the increase in IAAP was not dependent upon exogenously applied IAA alone. The increase in IAAP therefore may be regarded as a response to applied growth regulants in general and lends support to the concept that IAAP is naturally occurring in tomato and possibly in other species such as the soybean and tobacco grown on 8-hour photoperiods (11).

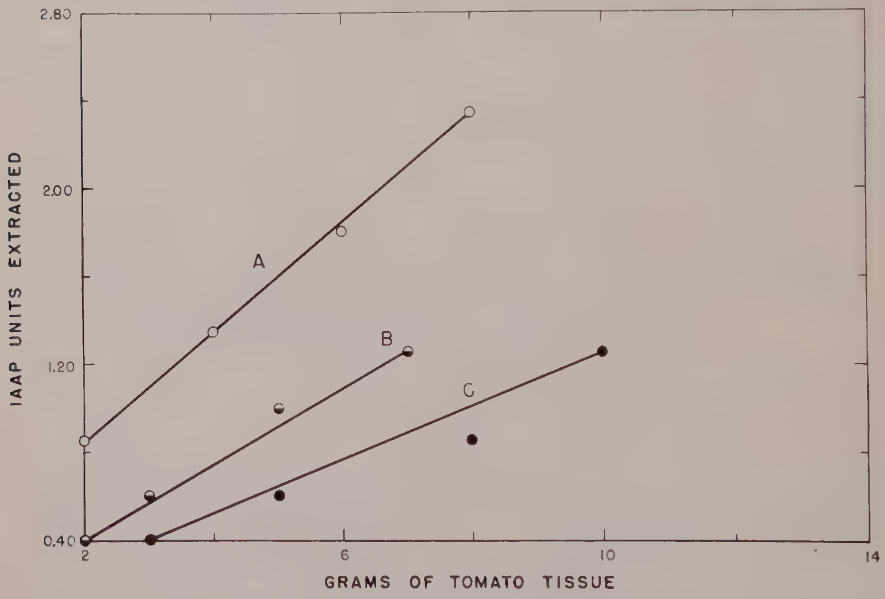

FIGURE 5. Relation between the amount of tomato tissue in extracts spotted on paper and the relative amount of IAAP in the extract. (A) Plants injected with 2.5 μg. per g. tissue and harvested 48 hrs. later. (B and C) Control plants in summer and winter, respectively. Five g. of tissue yielded one unit of IAAP (curve B). For method of determining IAAP units see Figure 1.

It was previously shown that a linear relationship existed between the fluorescence of the unidentified substance and the amount of tissue represented by chromatographic spots located at Rf 0.25 to 0.29 by means of an ultraviolet lamp (7). IAA located at Rf 0.54 on the same chromatogram showed a similar proportionality between the amount per chromatographic spot and fluorescence. Unfortunately, a synthetic sample of IAAP was not then available for comparison with the fluorescent substance in the ethanolic extract of control tomato tissue. However, fluorescence techniques should prove useful in future work dealing with the identification of IAAP or other fluorescent substances in extracts of plant tissue.

The results with tomato appear to show that naturally-occurring IAA as well as exogenously applied IAA conjugate with aspartic acid to form IAAP. Final proof would no doubt require the use of radioactive labeling to determine whether the extracted IAAP contained only the exogenously applied IAA. However, it is not understood why pretreatment of tomato plants with NAA and ethylene should cause a substantial increase in extractable IAAP, unless IAAP is part of a naturally-occurring system which regulates the level of IAA. The smaller increase resulting from 2,4-D pretreatment may not represent significant differences. The laboratory synthesis of a 2,4-D aspartic acid conjugate by Good (3) indicates that aspartic acid or possibly other organic acids may combine in plant tissue with exogenously applied 2,4-D or NAA to form conjugates similar to IAAP. If this occurs in tomato it is evident that the different conjugates could not be separated with the chromatographic procedures used in the present tests.

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NEMATOCIDAL PROPERTIES OF DIPHENYL SULFIDE[1]

DEWAYNE C. TORGESON[2] AND HARRY L. HAYNES[3]

SUMMARY

Diphenyl sulfide was found to have a high order of nematocidal activity when evaluated against the root-knot nematode (*Meloidogyne* sp.) and *Panagrellus redivivus*. In the laboratory, it was as effective as Nemagon (1,2-dibromo-3-chloropropane), Vapam (sodium *N*-methyldithiocarbamate dihydrate), Mylone (3,5-dimethyltetrahydro-2*H*-1,3,5-thiadiazine-2-thione), and ethylene dibromide and more effective than D-D (mixture of 1,3-dichloropropene and 1,2-dichloropropane) and PRD (3,4-dichlorotetrahydrothiophene-1,1-dioxide). In a North Carolina field test diphenyl sulfide gave good control of root-knot nematodes. Although the compound kills slowly, it has an approximate half-life in the soil of three weeks allowing sufficient time to reduce nematode populations as completely as faster acting materials. It has a low vapor pressure so is not effective unless intimately mixed with the soil, and, although only slightly water soluble, some movement in the soil water occurs. As diphenyl sulfide is phytotoxic to seed and young plants, it appears most promising as a preplant treatment.

INTRODUCTION

During the course of an investigation on the biological activity of various sulfur bridged organic compounds, diphenyl sulfide was found to be nematocidally active. Subsequent studies in the laboratory, greenhouse, and field showed that diphenyl sulfide is an effective, nonvolatile, residual type nematocide for the control of plant parasitic nematodes. A report of these studies is presented in this paper. Substituted diphenyl sulfide derivatives have also been recently reported to have nematocidal properties (1).

MATERIALS AND METHODS

Diphenyl sulfide samples used were either purchased from the Eastman Chemicals Company or prepared from benzene and sulfur dichloride and supplied by Dr. J. R. Kilsheimer, Research Department, Union Carbide Chemicals Company, South Charleston, West Virginia. Purified diphenyl sulfide is a colorless liquid but most samples were a light yellow to brown depending upon the amount of impurities present. These slight impurities did not detectably affect the compound's nematocidal activity. The compound has a typical sulfurous odor, a boiling point of 296 to 297° C., and its solubility in water is less than 0.01 per cent at 20° C.

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[1] These investigations were conducted under the terms of a fellowship established by Union Carbide Chemicals Company.

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Proprietary or experimental nematocidal materials used for comparative purposes were PRD (3,4-dichlorotetrahydrothiophene-1,1-dioxide), Vapam (sodium *N*-methyldithiocarbamate dihydrate), Nemagon (1,2-dibromo-3-chloropropane), D-D (mixture of 1,3-dichloropropene and 1,2-dichloropropane), ethylene dibromide, and Mylone (3,5-dimethyltetrahydro-2*H*-1,3,5-thiadiazine-2-thione).

Nematocidal activity was assessed using the root-knot nematode (*Meloidogyne* sp.) with cucumber (*Cucumis sativus* L. variety Chicago Pickling) or tomato (*Lycopersicon esculentum* Mill. var. Rutgers and Marglobe) as the indicator plants and by *in vitro* tests using the vinegar eelworm [*Panagrellus redivivus* (Linn. 1767) Goodey 1945]. Phytotoxicity and nematocidal effectiveness under field conditions were determined in tests conducted in New York and North Carolina.

LABORATORY AND GREENHOUSE TESTS

Root-knot nematode control. Inoculum was prepared by incubating for 1 week a mixture of tomato roots heavily infested with the root-knot nematode and composted loam soil. One teaspoon of the prepared inoculum was mixed with 180 cc. of soil in a pint jar. Ten ml. of a solution containing the test chemical was then added to each jar, the jar capped and rolled for five minutes to insure thorough mixing of the chemical and soil. After a holding period of 48 hours, the jars were uncapped and the contents placed in a 3-inch clay pot. The pots were seeded with 10 to 15 cucumber seeds and held in the greenhouse for 3 to 4 weeks at which time the roots of plants in untreated soil were heavily galled. The plants were then carefully removed and the amount of root galls was rated using a scale of 1 to 5 in which 1 equals severe; 2, moderate; 3, light; 4, very light; and 5, no galls. Application rates were calculated as pounds of active ingredient per six-inch acre on a volume basis and each treatment was thrice replicated. Similar experiments were also conducted using tomato as the indicator plant in larger clay pots with correspondingly greater volumes of soil.

Panagrellus test. Panagrellus redivivus cultures 10 days of age were exposed to test chemicals to measure contact activity over a period of time. The method of Tarjan (4) and Tarjan and Cheo (5) as modified by Schuldt and Bluestone (3) was employed. Contact mortality of diphenyl sulfide, Nemagon, PRD, and Mylone was determined at daily intervals in a concentration series of from 6.25 to 800 p.p.m.

FIELD TESTS

Nematode control and residual activity. The effect of diphenyl sulfide in comparison with Nemagon on the root-knot nematode was determined in a field trial at Clayton, North Carolina, on a Norfolk sandy loam. Plots were 20 × 20 feet replicated eight times in a randomized block design. Diphenyl sulfide and Nemagon were each applied at rates of 75, 37.5, and 18.7 lbs. of active ingredient per acre on May 21, 1958. Emulsifiable concentrate formulations of both materials were mixed with water and sprayed over the surface of the plots which were immediately double-disked to a depth of approximately six inches. Fifteen days following application of the test chemicals, four rows of Marglobe tomatoes were set in each plot. In late September, the plants were carefully uprooted and the amount of gall formation rated using the scale described above.

An indication of the residual activity of diphenyl sulfide was determined using samples of soil from field plots on a clay loam soil at Pine Brook, New Jersey, which had been treated as above except that diphenyl sulfide was applied at 80, 40, and 20 lbs. per acre and the plots were replicated five times. Samples collected immediately and at intervals up to 4 weeks following chemical application were mixed with a small amount of root-knot inoculum and seeded to cucumber. The roots were subsequently observed and the amount of gall formation rated. The number of galls formed was considered indicative of the amount of diphenyl sulfide remaining in the soil.

Phytotoxicity. Phytotoxicity of diphenyl sulfide to a number of seeded and transplanted crops was determined in duplicate tests conducted at Yonkers, New York, and Clayton, North Carolina. The chemical was applied as above at rates of 75, 37.5, and 18.7 lbs. per acre, and each treatment was replicated three times. Plantings were made one and fifteen days following chemical application by seeding corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), cucumber, and soybean (*Glycine max* Merr.), and setting transplants of tobacco (*Nicotiana tabacum* L.) and tomato in a 20-foot row in each plot. Three to four weeks later, the plots were observed and any differences in stand and vigor between treated and control plots noted.

EXPERIMENTAL RESULTS

LABORATORY AND GREENHOUSE TESTS

Root-knot nematode control. In a series of greenhouse tests, diphenyl sulfide was shown to be an effective nematocide for controlling root-knot nematodes on cucumber. The control obtained was comparable to that given by Nemagon, Vapam, Mylone, and ethylene dibromide and was superior to that of D-D and PRD (Table I). Similar results were also obtained when tomatoes were used as indicator plants.

The ability of diphenyl sulfide to penetrate and kill nematodes in infected roots was studied by modifying the root-knot test. Tomato seedlings were established in root-knot infested soil and allowed to grow for 3 weeks. The heavily galled plants were then removed, their roots washed, and transplanted to triplicated 5-inch clay pots containing noninfested soil which had been treated by incorporating diphenyl sulfide at rates of 150 and 75 lbs. per acre using a twin shell dry blender (Patterson-Kelley Co., Inc., Stroudsburg, Pa.). After being exposed to the chemical for 1 week, the plants were removed, the roots washed and equal portions of the galled roots from each pot chopped and mixed with noninfested soil in a 5-inch clay pot. A tomato seedling was transplanted into each pot and after 3 weeks was removed and the amount of gall formation observed. All treatments were as heavily galled as the controls indicating that diphenyl sulfide did not penetrate roots and kill established root-knot nematodes. Likewise, no nematode control was achieved when, in a similar experiment, diphenyl sulfide was applied as a soil drench at rates of 150 and 75 lbs. per acre to soil around heavily infested tomato plants in a test made in triplicate in 5-inch clay pots. However, an indication that diphenyl sulfide has some ability to penetrate tomato roots and kill root-knot nematodes was obtained when the roots of infected tomato seedlings were held in solutions of the chemical for 24 hours. Following the soaking period, the tomato seedlings were washed and planted in nematode-free soil. About three weeks later, the plants were cut off at the soil line and each pot replanted with a tomato seedling. Subsequent ratings of the root-knot present at rates of 100, 25, and 6.25 p.p.m. averaged 4.7, 5.0, and 1.3 for the three replications respectively.

TABLE I

CONTROL OF THE ROOT-KNOT NEMATODE ON CUCUMBER BY VARIOUS NEMATOCIDAL MATERIALS

Compound	Average root-knot rating* at indicated rate, lbs./A. active ingredient				
	150	75	37.5	18.7	9.4
Diphenyl sulfide	5.0	4.7	3.6	2.0	1.0
Nemagon	5.0	5.0	4.5	2.6	1.3
Vapam	5.0	5.0	5.0	3.0	1.0
Mylone	5.0	5.0	5.0	1.3	1.3
Ethylene dibromide	5.0	5.0	5.0	3.3	1.3
PRD	5.0	2.5	2.0	2.0	1.0
D-D	5.0	1.3	1.3	1.0	1.0

* Rating system used ranged from 1 for severe to 5 for no gall development.

Rate of kill. The mortality of *Panagrellus redivivus* exposed to various concentrations of diphenyl sulfide was found to increase with time (Table II). At the end of a one-day contact period, a concentration of 800 p.p.m. was necessary to obtain 100 per cent mortality whereas, after six days, 100 and 97 per cent were obtained at 100 and 25 p.p.m. respectively. Similar results were obtained with Nemagon and PRD. In contrast, the initial mortality obtained with Mylone was higher, but remained relatively unchanged with time. These results indicate that diphenyl sulfide, Nemagon, and PRD kill very slowly, whereas Mylone kills very rapidly. Using the same method but substituting root-knot nematode larvae obtained by aerating galled tomato roots in water, similar but more variable results were obtained.

Effect of application methods. Early tests showed that diphenyl sulfide gave better nematode control when intimately mixed with soil than when injected as is the usual practice with volatile nematocides. The standard nematocide evaluation method was, therefore, modified to compare the control obtained with several application methods in the greenhouse under conditions similar to those that might be used in the field. Half-gallon crocks with a hole in the bottom were filled with soil infested with root-knot nematodes and treated with diphenyl sulfide formulated as a 60 per cent active emulsifiable concentrate in a dosage series of 150, 75, 37.5, 18.7, and 9.4 lbs. per acre. The chemical was applied using the following methods of application: 1) thorough soil incorporation—chemical mixed thoroughly using the twin shell blender, 2) poor soil incorporation—surface

TABLE II

INACTIVATION RATE OF PANAGRELLUS REDIVIVUS BY DIPHENYL SULFIDE

Compound	Concn., p.p.m.	Percentage contact mortality at various days after exposure to test chemical					
		1	2	3	4	5	6
Diphenyl sulfide	800	100	—	—	—	—	—
	400	20	66	100	—	—	—
	100	13	28	93	100	—	—
	25	7	6	44	46	75	97
	6.25	8	5	7	12	14	14
Nemagon	800	3	5	59	71	83	100
	400	0	2	62	66	71	93
	100	5	4	8	27	27	52
	25	6	6	10	11	14	16
	6.25	7	7	5	10	14	17
PRD	800	100	—	—	—	—	—
	400	86	100	—	—	—	—
	100	5	7	56	70	100	—
	25	4	0	21	27	53	52
	6.25	2	3	17	20	18	20
Mylone	800	100	—	—	—	—	—
	400	100	—	—	—	—	—
	100	68	100	—	—	—	—
	25	0	6	15	16	18	20
	6.25	4	6	8	13	16	16
Control	—	4	6	7	7	12	15

applied chemical mixed poorly with three turns of a hand trowel, 3) surface application– surface applied chemical mixed into first inch of soil, 4) drench application –surface applied chemical drenched into soil with 400 ml. of water (sufficient to wet the soil to bottom of crock), and 5) soil injection –chemical applied with a syringe in a 3-inch deep hole in center of crock and the hole closed immediately afterwards. Three replications were used per treatment. After a holding period of three days at 75° F., a tomato seedling was planted in each crock and held for 3 weeks in the greenhouse. Observations of gall development showed that thorough soil incorporation of diphenyl sulfide was the best application method (Table III). The other methods were less effective, with soil injection being the poorest. Diphenyl sulfide apparently does not move to any great extent laterally or vertically in soil so good nematode control is not obtained unless the compound is thoroughly mixed with the soil.

TABLE III

EFFECT OF VARIOUS APPLICATION METHODS ON NEMATODE CONTROL BY DIPHENYL SULFIDE

Method of application	Root-knot ratings* at several rates of application (lbs./A.)				
	150	75	37.5	18.7	9.4
Thorough soil incorporation	5.0	4.5	3.5	3.0	2.0
Poor soil incorporation	4.5	3.0	1.5	1.5	2.0
Surface incorporation	3.5	3.0	2.5	2.5	1.5
Drench	3.5	3.0	2.5	1.5	1.5
Injection	2.5	1.5	1.5	1.5	1.5

* See footnote, Table I.

As drenching was considered a possible means of applying diphenyl sulfide to established plants, the effect of varying amounts of drenching water on nematode control was studied. Diphenyl sulfide was applied at rates of 75, 37.5, and 18.7 lbs. per acre to the soil surface of a series of half-gallon glazed crocks filled with root-knot infested soil. Three crocks at each concentration were then drenched with one, two, or four acre-inches of water and, after being held overnight, were seeded with cucumber. Subsequent observations on the number of galls present on the roots of plants in the various treatments showed that as the amount of water applied increased, the number of galls decreased (Table IV).

FIELD EVALUATION

Nematode control. Tomatoes in plots treated with diphenyl sulfide at 75, 37.5, and 18.7 lbs. per acre had significantly less root knot than those in untreated plots (Table V) when the data were analyzed using Wil-

TABLE IV

EFFECT OF DRENCHING UPON THE NEMATODE CONTROL OF SURFACE APPLIED DIPHENYL SULFIDE

Application rate, lbs./A.	Root-knot ratings* at different drenching levels (acre-inches)		
	4	2	1
75	4.7	4.3	4.0
37.5	4.3	3.7	3.3
18.7	4.0	3.0	2.7
Control	1.7	1.7	1.0

* See footnote, Table I.

coxon's rank method (6). No significant differences were found between the incidence of root knot in the Nemagon plots at all rates and untreated plots. Although root-knot damage was not severe in the control plots, these data indicate that at rates as low as 18.7 lbs. per acre diphenyl sulfide is an effective nematocide for the control of the root-knot nematode. Similar tests conducted a year later were less conclusive as the extreme plot-to-plot variability did not permit the detection of differences between treated and untreated plots.

Residual activity. Examination of the data, obtained when the residual nematocidal activity present in soil which had been treated with diphenyl sulfide at rates of 80, 40, and 20 lbs. per acre was determined by inoculating samples at intervals with the root-knot nematode and assaying the control obtained on cucumber roots, shows that diphenyl sulfide has a half-life in this particular soil of approximately 3 weeks (Table VI). As only light showers fell during this period, no appreciable amount of diphenyl sulfide could have been lost due to leaching but rather must have been

TABLE V

EFFECTIVENESS OF DIPHENYL SULFIDE AND NEMAGON IN CONTROLLING THE ROOT-KNOT NEMATODE ON TOMATO IN THE FIELD

Compound	Rate, lbs./A.	Average root-knot ratings* in replicate tests								
		1	2	3	4	5	6	7	8	Av.
Diphenyl sulfide	75	5.00	5.00	4.17	5.00	4.75	4.80	4.86	5.00	4.82**
	37.5	4.67	4.55	3.50	5.00	4.88	4.40	5.00	4.38	4.55**
	18.7	5.00	5.00	4.73	4.56	4.50	4.33	4.80	5.00	4.74**
Nemagon	75	5.00	5.00	4.25	4.82	2.30	4.89	4.38	—	4.37
	37.5	5.00	4.25	1.80	3.30	3.88	5.00	3.00	4.75	3.87
	18.7	4.18	4.55	1.64	4.00	4.36	3.33	4.10	3.29	3.68
Control	—	4.92	2.00	1.64	3.00	3.20	3.40	4.29	2.00	3.06

* See footnote, Table I.
** Significantly less galling than the control at the 5 per cent significance level.

lost due to decomposition or inactivation in the soil. Although this is a relatively crude means of determining residual activity, the results are in good agreement with those obtained more recently when the amount of material present was determined using an analytical procedure which will be described in a subsequent paper.

Phytotoxicity. Diphenyl sulfide at rates of 75, 37.5, and 18.7 lbs. per acre was slightly injurious to corn, cotton, cucumber, soybean, tobacco, and tomato seeded or transplanted the day following treatment. Injury was somewhat more pronounced on the sandy loam soil in North Carolina than on the silt loam soil in New York. Plantings made 15 days after treatment were free of injury at both locations. Two weeks appeared to be a safe interval between treatment and planting with these crops. Depending upon susceptibility to damage from these chemicals, a shorter or longer interval would perhaps be required with other crops.

TABLE VI

RESIDUAL ACTIVITY OF DIPHENYL SULFIDE AS INDICATED BY THE CONTROL OF THE ROOT-KNOT NEMATODE OBTAINED IN REINFESTED SOIL

Rate, lbs./A.	Root-knot rating* in soil reinfested at intervals following treatment					
	Immediately	3 Days	1 Week	2 Weeks	3 Weeks	4 Weeks
80	4.5	4.7	4.3	4.5	3.7	2.1
40	3.9	4.0	3.9	2.5	2.9	1.7
20	3.5	3.6	3.6	2.9	1.9	1.3
Control	2.1	2.2	2.2	1.8	1.5	1.2

* See footnote, Table I.

Diphenyl sulfide drenched around established roses (*Rosa multiflora* Thunb. rootstock), box (*Buxus sempervirens* L.), and privet (*Ligustrum vulgare* L.) at rates up to 80 lbs. per acre caused no plant injury indicating that it could be safely applied to established woody plants.

DISCUSSION

Diphenyl sulfide has been demonstrated to be an effective nematocide in laboratory and preliminary field evaluations at dosage levels at or below those of commonly used commercial nematocides. Diphenyl sulfide, however, has a low vapor pressure and does not perform satisfactorily when applied by soil injection in the manner used for the volatile nematocides. It must, therefore, be incorporated into the soil as intimately as possible if maximum nematode control is to be obtained. This can be achieved by applying the material to the soil surface, as a liquid spray or adsorbed on a carrier, followed by thorough disking or rotary tilling. Although diphenyl sulfide is only slightly water soluble, it does move to a limited extent in the soil water. As the material is not volatile, local movement in soil water may well be essential for the obtainment of good nematode control. Diphenyl sulfide like Nemagon and PRD (2, 3) is slow acting but as it is sufficiently residual the same level of nematode control can be achieved as with faster acting materials.

Like most nematocides, diphenyl sulfide is toxic to seeds and seedlings necessitating its use one to two weeks prior to planting. The waiting period necessary to avoid phytotoxicity will, of course, vary depending upon the amount of diphenyl sulfide applied, soil type, rainfall, and other environmental factors. Perennial crops appear to be more tolerant but as the compound does not readily penetrate roots and could not be easily applied to established crops, diphenyl sulfide appears to offer most promise as a preplant nematocide.

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THE FUNGICIDAL ACTIVITY OF (AMINOALKYL)PYRENES[1]

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SUMMARY

The (aminoalkyl)pyrenes were found to be active fungicides when toxicity was assayed in the spore germination test using *Alternaria oleracea* and *Monilinia fructicola* and in the control of tomato early blight (*Alternaria solani*), bean powdery mildew (*Erysiphe polygoni*), and apple powdery mildew (*Podosphaera leucotricha*). Pyrene, the parent compound, is active only against the powdery mildews. *N*-methyl-1-pyrenemethylamine was the most effective compound for early blight control and *N,N*-dimethyl- and *N,N*-diethyl-1-pyrenemethylamine hydrochloride were the best for powdery mildew control. In general, activity decreased as the *N*-alkyl chain was lengthened. Heterocyclic amine derivatives were also less toxic than the lower members of the *N*-alkyl series. Phytotoxicity was particularly severe among the more active compounds.

INTRODUCTION

With the exception of their quinoid derivatives (6), polycyclic hydrocarbon compounds have been little investigated as fungicides. During the course of a study on the fungitoxicity of the pyrenes, it was found that the (aminoalkyl)pyrenes were active fungicides. The biological activities of a series of these compounds are discussed in this report. Their synthesis has been reported elsewhere (3).

MATERIALS AND METHODS

Fungitoxicity of the compounds investigated was determined using spore inhibition, foliage protection, and foliage disease eradication techniques. Phytotoxicity to foliage was also measured. The author is indebted to Dr. E. Marcus, Research Department, Union Carbide Chemicals Company, South Charleston, West Virginia for providing the test chemicals.

Inhibition of spore germination of *Alternaria oleracea* Milb. and *Monilinia fructicola* (Wint.) Honey was measured by the test tube dilution method (1). Solutions or suspensions of the test chemicals were prepared at an initial concentration of 1000 p.p.m. by the addition of a solvent such as acetone and an emulsifier (Tergitol NPX, an alkyl phenyl polyethylene glycol ether) to give a final concentration of 5 and 0.01 per cent respectively. The ED50 ranges were determined using a tenfold dilution series and given an alphabetical rating (8) in which A equals 1 to 10; B, 10 to 100; C, 100 to 1000; and D, >1000 p.p.m.

Test chemicals for all foliage tests were formulated by preparing a stock solution or suspension of 4000 p.p.m. containing acetone at 20 per cent and emulsifier (Triton X-155, an alkyl aryl polyether alcohol) at 0.05 per cent. Aliquots of this stock were then diluted to obtain the desired concentration of chemical for the various spray tests.

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[1] These investigations were conducted under the terms of a fellowship established by Union Carbide Chemicals Company.

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Control of early blight, *Alternaria solani* (Ell. & Mart.) Jones & Grout, of tomato, *Lycopersicon esculentum* Mill., was used to assess the protective fungicidal ability of the test chemicals. The method was essentially that of McCallan (4) and McCallan and Wellman (5) except that disease control was rated at each concentration of chemical using a rating system of 1 to 5. In this system, 0 to 24 per cent control was represented by 1; 25 to 49 per cent by 2; 50 to 69 per cent by 3; 70 to 89 per cent by 4; and 90 to 100 per cent by 5. Zineb, freshly prepared from nabam (sodium ethylenebis[dithiocarbamate]) and zinc sulfate, was used as a standard fungicide in all tests.

For the powdery mildew eradication test, bean plants, *Phaseolus vulgaris* L. var. Tendergreen, grown in 4-inch clay pots were inoculated by dusting with spores of the powdery mildew fungus, *Erysiphe polygoni* DC., when the primary leaves were fully expanded. Two days later, the plants were sprayed with the test chemicals at the desired concentrations and after spraying were held in the greenhouse for 7 to 10 days. The amount of mildew on the primary leaves was then rated using a system in which 1 represented no control (leaf covered with mildew); 2, many discrete spots; 3, a moderate number of spots (4 to 10) per leaf; 4, a few spots (1 to 3) per leaf; and 5, no infection. Powdery mildew, *Podosphaera leucotricha* (Ell. & Ev.) Salmon, on seedling apple, *Pyrus malus* L. var. McIntosh, was also used in the evaluation of these compounds for protective fungicidal activity (7). Karathane (2-[1-methylheptyl]-4,6-dinitrophenyl crotonate), a commercial powdery mildew fungicide, was included in these tests for comparative purposes.

Foliage phytotoxicity was determined on bean plants with their first set of trifoliate leaves expanded and on tomato and corn (*Zea mays* L.) plants 6 inches high. The plants were sprayed with the test chemicals at a concentration of 1000 p.p.m. One week later, the amount of foliar injury was visually estimated and given a score from 1 for no injury to 5 for very severe injury.

EXPERIMENTAL RESULTS

The names, substituents on the pyrene nucleus, and the ratings obtained for the various derivatives in the spore-germination, tomato early blight, bean powdery mildew, apple powdery mildew, and phytotoxicity tests are given in Table I.

Spore germination inhibition and tomato early blight control data for the various compounds were in fair agreement, with greater differences between chemicals being found in the early blight test. Pyrene, the parent compound, was inactive, but when substituted with a methylamine group fungicidal activity was obtained. Alkyl substitutions on the amine group markedly influenced the degree of early blight control obtained. The *N*-methyl derivative was the most active member of the series with a gradual diminution of activity above diethyl to the *N*,*N*-di-(2-ethylhexyl) derivative which was essentially inactive. No clear-cut differences between the free base and hydrochloride were found in the two instances where both derivatives were available for evaluation. Heterocyclic amine derivatives, i.e., those of pyrrolidine, piperidine, morpholine, and piperazine were slightly less toxic than the lower members of the *N*-alkyl series. The *N*-phenyl and *N*-(2-pyridyl) derivatives were inactive. Marked differences in toxicity could not be detected between the α-aminoethyl and aminomethyl compounds and their *N*-derivatives.

Differences in fungitoxicity were less striking when the compounds were evaluated for apple and bean powdery mildew control. The *N*,*N*-dimethyl- and *N*,*N*-diethyl-1-pyrenemethylamine hydrochlorides were the most effective compounds in both tests and equal to the Karathane standard. The poorest control was obtained with unsubstituted pyrene. The phytotoxicity of these compounds at 1000 p.p.m. to bean, corn, and tomato varied considerably. With the exception of pyrene which was very phytotoxic, the more fungicidal compounds were the most phytotoxic.

DISCUSSION

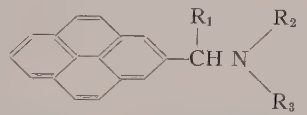
The (aminoalkyl)pyrenes are toxic to both the powdery mildews and other fungi such as *Alternaria* and *Monilinia*. Pyrene and a variety of derivatives which have been previously evaluated in this laboratory are much more specific with activity restricted to the powdery mildews. The aminoalkyl moiety apparently imparts to the molecule the proper balance between lipid and water solubilities necessary for penetration of the cell membranes of fungi other than the powdery mildews. Toxicity to the powdery mildrews is also enhanced.

The mechanism of action of the (aminoalkyl)pyrenes was not studied, but Fellig and Brough (2) have reported that in work with yeast (*Saccharomyces cerevisiae*) inhibition of growth could be overcome by the addition of deoxyribonucleic acid to the medium. Deoxynucleotides and deoxynucleosides obtained by enzymatically degrading deoxyribonucleic acid were also effective.

The phytotoxicity of the (aminoalkyl)pyrenes, especially when applied successively as would be done in field applications, prevents their use as commercial fungicides. They are, however, a new group of fungitoxic materials, and the information presented here may be useful in designing other biologically active molecules.

TABLE I

Fungicidal Effectiveness of a Group of (Aminoalkyl)pyrenes



Compound	Substituents		Spore inhibition ED50 range*		Disease control rating at chemical concentration p.p.m. of										Foliage phytotoxicity at 1,000 p.p.m.††		
					Tomato early blight**				Bean powdery mildew†				Apple powdery mildew†				
	R_1	N R_2R_3	*A. oleracea*	*M. fructicola*	1,000	100	20	4	500	100	20	4	20	4	Bean	Corn	Tomato
Pyrene	—	—	D	D	3				5	3	1	1			3	4	4
1-Pyrenemethylamine hydrochloride	H	NH_2	A	A	4	1			5	5	5	3	3		1	1	1
N-Methyl-1-pyrenemethylamine	H	$NHCH_3$	A	A	5	5	4	3	5	4	4	3	3		4	2	5
N-Methyl-1-pyrenemethylamine hydrochloride	H	$NHCH_3$	A	A	5	5	3		5	5	3	3	3		1	1	1
N,N-Dimethyl-1-pyrenemethylamine hydrochloride	H	$N(CH_3)_2$	A	A	4	3			5	5	4	3	5	4	3	2	4
N,N-Diethyl-1-pyrenemethylamine hydrochloride	H	$N(C_2H_5)_2$	B	A	5	5	3		5	5	4	3	5	5	4	2	4
N-Butyl-1-pyrenemethylamine	H	NH(*n*-C_4H_9)	A	A	4	3			5	5	3	3	2		1	1	2
N-Butyl-1-pyrenemethylamine hydrochloride	H	NH(*n*-C_4H_9)	A	B	5	3			5	5	4	3	3		1	1	2
N,N-Dibutyl-1-pyrenemethylamine hydrochloride	H	N(*n*-C_4H_9)$_2$	B	B	5	3			5	5	4	3	3		3	3	3

* A = 1–10, B = 10–100, C = 100–1,000, D = >1,000 p.p.m.
** 1 = 0–24, 2 = 25–49, 3 = 50–69, 4 = 70–89, 5 = 90–100 per cent disease control.
† 1 = No control, 2 = many but discrete spots, 3 = 4–10 spots per leaf, 4 = 1–3 spots per leaf, 5 = no mildew.
†† 1 = No injury, 2 = slight injury, 3 = moderate injury, 4 = severe injury, 5 = plant dead.

TABLE I (*Continued*)

Compound	Substituents		Spore inhibition ED_{50} range*		Disease control rating at chemical concentration p.p.m. of										Foliage phytotoxicity at 1,000 p.p.m.††		
					Tomato early blight**				Bean powdery mildew†				Apple powdery mildew†				
	R_1	$N\ R_2R_3$	*A. oleracea*	*M. fructicola*	1,000	100	20	4	500	100	20	4	20	4	Bean	Corn	Tomato
N,*N*-Dihexyl-1-pyrenemethylamine hydrochloride	H	$N(n\text{-}C_6H_{13})_2$	B	B	3	2			5	4	4	2	4	2	2	1	3
N,*N*-Diallyl-1-pyrenemethylamine hydrochloride	H	$N(CH_2CH{=}CH_2)_2$	B	B	4	2			5	4	3	2			1	1	1
N,*N*-Di-(2-ethylhexyl)pyrenemethylamine hydrochloride	H	$N[CH_2CH(C_2H_5)C_4H_9]_2$	D	C	2	2			5	5	4	1	3		1	1	2
N-(1-Pyrenylmethyl)pyrrolidine hydrochloride	H	$N(CH_2)_4$	A	A	4	5	2		5	5	4	3	4	3	2	1	3
N-(1-Pyrenylmethvl)piperidine hydrochloride	H	$N(CH_2)_5$	A	A	5	3			5	4	4	3	4	3	2	1	2
N-(1-Pyrenylmethyl)morpholine hydrochloride	H	$N(CH_2CH_2)_2O$	A	A	3	1			5	4	4	2	4	2	2	1	4
N-(1-Pyrenylmethyl)-*N*′-methyl-piperazine dihydrochloride	H	$N(CH_2CH_2)_2NCH_3$	A	B	4	4	2		5	4	4	3	3		2	1	3
N-Phenyl-1-pyrenemethylamine	H	NHC_6H_5	D	D	3	1			5	5	3	2	2		1	1	1
N-(2-Pyridyl)-1-pyrenemethylamine	H	$NH(\alpha\text{-pyridyl})$	D	C	1	1			5	3	2	2	2		1	1	1
1-(α-Aminoethyl)pyrene hydrochloride	CH_3	NH_2	A	A	5	2			5	5	4	2	4	2	2	2	1
1-(α-*N*-Methylaminoethyl)pyrene hydrochloride	CH_3	$NHCH_3$	A	A	4	5	4	1	5	5	4	4	3		2	1	2
1-(α-*N*-Hexylaminoethyl)pyrene hydrochloride	CH_3	$NH(n\text{-}C_6H_{13})$	A	A	4	2			5	5	4	3	3		2	1	3
Nabam					5	5	5	3									
Karathane									5	5	5	3	5	4			

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POLYARACYCLIC METHYLMERCAPTOIMIDAZOLINES AS FOLIAGE FUNGICIDES[1]

DEWAYNE C. TORGESON,[2] W. H. HENSLEY,[3] AND J. A. LAMBRECH[3,4]

SUMMARY

Inhibition of spore germination and control of early blight of tomato, powdery mildew of bean, and powdery mildew of apple assay techniques were used to study the fungicidal effectiveness of a group of polyaracyclic methylmercaptoimidazolines. The two most active compounds studied were 2-(3-fluoranthenylmethylmercapto)imidazoline hydrochloride and 2-(9-phenanthrylmethylmercapto)imidazoline hydrochloride. The free bases were less toxic. Anthracene, pyrene, and naphthalene derivatives were poorer fungicides than the fluoranthene and phenanthrene derivatives.

INTRODUCTION

During an investigation of the fungicidal properties of a variety of polyaracyclic compounds, it was found that several of the methylmercaptoimidazoline derivatives were effective fungicides. The fungicidal properties of imidazoline derivatives have been known for several years (14, 18) and glyodin (2-heptadecyl-2-imidazoline acetate) has been developed as a commercial fungicide. The imidazolines are, however, ineffective against the powdery mildews. In this respect, the polyaracyclic methylmercaptoimidazolines discussed in this report differ since they are toxic to the powdery mildews as well as to other fungi.

MATERIALS AND METHODS

PREPARATION OF COMPOUNDS

Methylmercaptoimidazoline substituted derivatives of fluoranthene, anthracene, pyrene, naphthalene, and phenanthrene were synthesized by first preparing the chloromethyl polyaracyclic compound (4, 5, 8) followed by a reaction with ethylenethiourea (7; 13, p. 31) to yield the desired compound. For example, 2-(3-fluoranthenylmethylmercapto)imidazoline hydrochloride was made as shown at the top of the next page.

Using the fluoranthene derivative as an example, 3-chloromethylfluoranthene was prepared in a 500-ml. four-neck reaction flask, equipped with a stirrer, thermometer, reflux condenser, diffuser for feeding hydrogen chloride beneath the surface of the reactor contents, and an electric heating mantle to supply external heat. The reaction was carried out by charg-

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[1] These investigations were conducted under the terms of a fellowship established by Union Carbide Chemicals Company.

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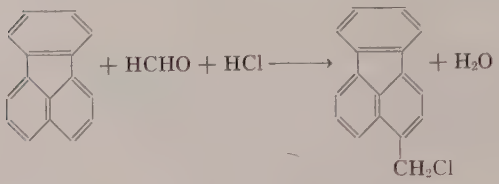
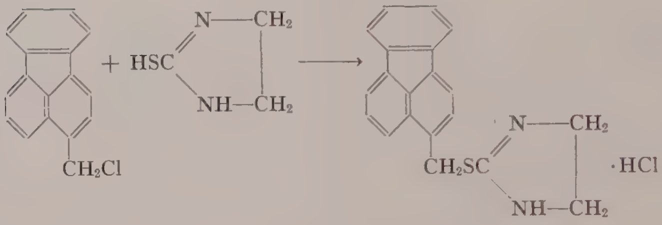


(*1*)



(*2*)

ing the reactor with 50 ml. of concentrated hydrochloric acid, 8 grams (0.25+mole) of paraformaldehyde, 60 ml. of glacial acetic acid, and 30 ml. of 85 per cent phosphoric acid; heating the mixture with stirring to 50° C. and at this point adding portionwise 51 grams (0.25 mole) of fluoranthene. After the mixing was complete, the mixture was heated at 75° to 80° C. for seven hours. An excess of gaseous hydrogen chloride was added throughout the heating period. The crude product was extracted with hot benzene. Upon recrystallization from benzene, a 60 per cent yield of 3-chloromethyl fluoranthene was obtained. The product had a melting point of 139° C. and was 98.5 per cent pure by elemental analysis.

2-(3-Fluoranthenylmethylmercapto)imidazoline hydrochloride was prepared in a 500-ml. four-neck reaction flask equipped with a stirrer, thermometer, reflux condenser, and an electric mantle for external heating. The flask was charged with 25 grams (0.1 mole) of 3-chloromethylfluoranthene, 10.2 grams (0.1 mole) of ethylenethiourea, and 300 ml. of ethanol. The mixture was then heated at the reflux point (about 80° C.) for five hours, cooled to room temperature, diluted with an equal volume of ethyl ether and filtered. Thirty grams of product were recovered. The other imidazoline hydrochlorides were made in a similar fashion. The free bases were prepared by reacting the imidazoline hydrochloride in water with an equimolar quantity of sodium hydroxide and isolating the product by filtration.

BIOLOGICAL EVALUATION

Fungitoxicity of the compounds was measured using inhibition of spore germination foliage disease eradication, and foliage protection evaluation techniques in the greenhouse and laboratory.

The test tube dilution method (1) was used to determine the inhibition of spore germination of *Alternaria oleracea* Milb. and *Monilinia fructicola* (Wint.) Honey. Test chemicals were formulated as solutions or suspensions in water at an initial concentration of 1,000 p.p.m. by adding acetone and emulsifier (Tergitol NPX, an alkylphenyl polyethylene glycol ether) at 5.0 and 0.01 per cent respectively. A tenfold dilution series was used in determining the ED50 values. Those with an ED50 in the range of 1 to 10 p.p.m. were designated as A, 10 to 100 p.p.m. as B, 100 to 1,000 p.p.m. as C, and $>$1,000 p.p.m. as D (17).

The method of McCallan (9) and McCallan and Wellman (10) was used to evaluate effectiveness of the test chemicals in controlling early blight, *Alternaria solani* (Ell. & Mart.) Jones & Grout, of tomato, *Lycopersicon esculentum* Mill. Test chemicals for this and other foliage tests were formulated for spraying as a 4,000 p.p.m. stock solution or suspension containing 20 per cent acetone and 0.05 per cent emulsifier (Triton X-155, an alkyl aryl polyether alcohol). The stock was then diluted to the desired concentrations for spraying. Disease control was rated using a system of 1 to 5, in which 1 represented 0 to 24 per cent control; 2, 25 to 49; 3, 50 to 69; 4, 70 to 89; and 5, 90 to 100.

The chemicals were also evaluated as eradicants for the control of the powdery mildew, *Erysiphe polygoni* DC., of bean, *Phaseolus vulgaris* L. (15). The most active compounds in this test were then tested as protectants for the control of the powdery mildew, *Podosphaera leucotricha* (Ell. & Ev.) Salmon, of apple, *Pyrus malus* L. (16). In both tests the degree of disease control was measured by rating the amount of mildew present using a system in which 1 represented no control (leaf covered with mildew); 2, many discrete spots; 3, a moderate number of spots (4 to 10) per leaf; 4, a few spots (1 to 3) per leaf; and 5, no infection.

EXPERIMENTAL RESULTS

The compounds evaluated and the ratings obtained in the spore germination, tomato early blight, bean powdery mildew, and apple powdery mildew tests are given in Table I.

Fungicidal activity of the compounds was in general well correlated in the various tests. The two most toxic compounds were 2-(3-fluoranthenylmethylmercapto)imidazoline hydrochloride and 2-(9-phenanthrylmethylmercapto)imidazoline hydrochloride. The free bases of these two compounds were less active. When the imidazoline group of the fluoranthene derivative was substituted in the 1 position with a 2-hydroxyethyl group, activity was reduced. The anthracene, pyrene, and naphthalene derivatives were the least toxic members of this series.

DISCUSSION

The polyaracyclic methylmercaptoimidazolines combine two fungicidal groups to yield compounds with spectra of activity different from either parent compound. The imidazolines (14, 18) and ethylenethioureas

TABLE I

FUNGICIDAL EFFECTIVENESS OF A GROUP OF POLYARACYCLIC METHYLMERCAPTOIMIDAZOLINES

Compound	Spore inhibition ED50 range*		Disease control rating at chemical concentration (p.p.m.) of											
			Tomato early blight**				Bean powdery mildew†				Apple powdery mildew†			
	A. oleracea	*M. fructicola*	400	80	16	3.2	400	80	16	3.2	400	80	16	4
2-(3-Fluoranthenylmethylmercapto)-imidazoline	B	A	5	4	3		5	4	3		5	4	4	2
2-(3-Fluoranthenylmethylmercapto)-imidazoline hydrochloride	A	A	5	5	3	1	5	5	4	3	5	5	4	3
2-(3-Fluoranthenylmethylmercapto)-1-(2-hydroxyethyl)imidazoline hydrochloride	C	B	3	2			5	3	1					
2-(9-Anthrylmethylmercapto)imidazoline hydrochloride	C	B	4	3			5	4	2					
2-(9-Phenanthrylmethylmercapto)-imidazoline	C	B	5	4	1		3	1						
2-(9-Phenanthrylmethylmercapto)-imidazoline hydrochloride	A	A	5	5	4	1	5	4	3		5	4	3	
2-(3-Pyrenylmethylmercapto)imidazoline hydrochloride	C	C	4	3			5	5	2					
2-(2-Naphthylmethylmercapto)imidazoline hydrochloride	B	B	5	3	1		3	1						

* A = 1–10, B = 10–100, C = 100–1,000, D = >1,000 p.p.m.

** 1 = 0–24, 2 = 25–49, 3 = 50–69, 4 = 70–89, 5 = 90–100 per cent disease control.

† 1 = No control, 2 = many but discrete spots, 3 = 4–10 spots per leaf, 4 = 1–3 spots per leaf, 5 = no mildew

(12) although highly fungicidal are not active against the powdery mildews, whereas the polyaracyclics except quinones (11) and (aminoalkyl)pyrenes (15) are toxic to the powdery mildews, but are relatively nontoxic to other fungi. Combination of the two toxic groups apparently results in a compound which has physico-chemical properties that make it toxic to a greater variety of fungi. Unfortunately, insufficient information on the biochemistry and chemical composition of the different fungi is available to allow speculation as to the factors involved.

From among the compounds evaluated, 2-(3-fluoranthenylmethylmercapto)imidazoline hydrochloride was selected as the most promising and was tested in the field by a number of cooperators, coded as Compound 10521. It was found to be a fairly effective fungicide against the powdery mildews (2, 3, 6). However, the compound was somewhat phytotoxic which has precluded the development of 2-(3-fluoranthenylmethylmercapto)imidazoline hydrochloride as a commercial product.

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Note

A GREENHOUSE METHOD FOR EVALUATION OF CHEMICALS TO CONTROL APPLE POWDERY MILDEW[1]

Dewayne C. Torgeson[2] and C. G. Lindberg[3]

The general replacement of sulfur by organic fungicides such as glyodin and captan for the control of apple scab in recent years has resulted in an increased incidence of powdery mildew, *Podosphaera leucotricha* (Ell. & Ev.) Salmon, in the major apple growing areas of the United States. A suitable greenhouse screening method for the selection of active compounds for field evaluation was desirable if new fungicides were to be found and developed for the control of this disease. Budded apple trees in pots, although usable for the evaluation of powdery mildew fungicides, were found to be unsuitable test plants because of the space required and the unavailability of actively growing plants throughout the year. A satisfactory method was developed by modifying that described by Rich and Richards (4) in which apple seedlings were used for the evaluation of fungicides for the control of apple scab. A description of the modified method and a discussion of its usefulness is reported in this paper.

Plants are grown by sowing seed from McIntosh apples in flats of moist white silica sand. When the emerged seedlings have developed 2 to 4 true leaves, they are transplanted to $3\frac{1}{2}$-inch clay pots containing a light composted soil. Plants can be used for testing when at least six leaves have developed. This stage is usually reached five to six weeks from the time of seeding. Test plants can be reused by cutting them back to 1 or 2 buds above the soil line and allowing one shoot to develop. By periodic seeding and reuse of plants, a continuous supply of test plants can be maintained.

As only young leaves are susceptible to the powdery mildew fungus, it is of utmost importance to keep test plants healthy and actively growing. Frequent applications of a soluble fertilizer such as Miracle-Grow (Stern's Nurseries, Inc., Geneva, N. Y.) applied either to the soil or foliage is helpful in maintaining a steady rate of growth. Mites, aphids, and powdery mildew are also frequent problems and must be controlled if succulent growth is to be maintained. Aphids are readily eliminated by spraying with Malathion [*O*,*O*-dimethyl-*S*-(1,2-dicarbethoxyethyl)dithiophosphate]. Mites and powdery mildew can be satisfactorily controlled by growing the plants in a fine water mist from misting nozzles such as are commonly used in plant propagation beds.

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[1] These investigations were conducted under the terms of a fellowship established by Union Carbide Chemicals Company.

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Chemicals may be evaluated either as protectants or eradicants. Plants of equal size and vigor are selected for testing. Three plants are used in each treatment. In the protectant test, plants are placed on a compound turntable and sprayed with the test chemical in the same fashion as in the tomato foliage disease test (2, 3). They are then continuously exposed to powdery mildew spores by intermixing on a greenhouse bench with heavily infected apple seedlings. One week later, they are resprayed and returned to the greenhouse bench. Ten days after the second spraying, the amount of mildew infection is rated using a system in which 1 represents no control (leaf covered with mildew); 2, many discrete mildew spots; 3, a moderate number of spots (4 to 10) per leaf; 4, a few spots (1 to 3) per leaf; and 5, no infection. Eradicant tests are conducted in a similar manner except that test plants are exposed to mildew spores for ten days prior to spraying and only one spraying is used. The amount of sporulating mildew is rated in another 10 days using the system described above. Results of a large number of repeated tests show that the ratings for a particular compound do not vary more than ± 1 from test to test in either the eradicant or protectant test.

This method has been very useful in the evaluation of a large number of candidate compounds and the selection of the more effective ones for field evaluation. Limited experience indicates that correlation between the control obtained with a chemical in this test and in field tests is good. For example, when 1-nitropyrene was compared with Karathane [2-(1-methylheptyl)-4,6-dinitrophenyl crotonate], a commercial powdery mildew fungicide, at concentrations of 100, 20, and 4 p.p.m., average ratings of 5.0, 3.2, and 1.0, respectively, were obtained as compared with 5.0, 4.3, and 2.0 for Karathane. Evaluated in the field under code (Compound 9688), 1-nitropyrene gave mildew control only slightly inferior to that obtained with Karathane (1).

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Note

THE PRESENCE OF SEDOHEPTULOSE IN COLEUS BLUMEI BENTH.

PHILIP H. PLAISTED

The presence of a seven carbon keto sugar, sedoheptulose, has been demonstrated in a number of plant species (3, 4, 5, 6). The evidence for its existence is mainly chromatographic although La Forge and Hudson (2) did purify the compound and form the anhydride from *Sedum spectabile;* later Hudson (1) confirmed the structure. Tolbert *et al.* (7) have presented chromatographic evidence of sedoheptulose in coleus. Recent studies in this laboratory have confirmed the work of Tolbert *et al.* (7) and indicate that sedoheptulose is the predominating carbohydrate in coleus. Its crystallization as the anhydride and proof of its identity are presented here.

Sedoheptulose, similar to many naturally occurring sugars, does not crystallize when extracted and purified; therefore, the compound was extracted as the anhydride rather than the natural sugar. The anhydride was obtained by extracting one kilo of fresh coleus leaves with 80 per cent ethanol. The alcohol was evaporated and the water phase concentrated to approximately 35 ml. of a syrupy liquid. The sedoheptulosan was formed directly in the syrup by the method outlined by La Forge and Hudson (2). Ten ml. of the syrup was diluted with 46 ml. of a one per cent HCl solution and refluxed for an hour on the steam bath. The cooled solution was made neutral to pH indicator paper with silver carbonate and the excess silver precipitated with hydrogen sulfide. After the precipitate had been filtered, it was necessary to boil the filtrate in order to precipitate a colloidal material which was removed by further filtration. The neutral solution was concentrated to a syrup and placed in the refrigerator to crystallize.

At the end of 72 hours, the liquid had solidified into a red crystalline mass. The color was caused by the anthocyanins present in the extract. The sample was purified by recrystallizations from 95 per cent ethanol until white crystals were obtained.

Identification of the unknown was carried out by comparing an infrared spectrum and melting point with authentic samples obtained from Nutritional Biochemical Company (N. B. C.). The infrared spectra were run in a KBr pellet on the Model 221 Perkin-Elmer recording spectrophotometer. The spectra obtained for the plant extract and the authentic sample were identical (Fig. 1).

Initially, the melting points of both the sedoheptulosan crystallized from the plant extract and that purchased from N. B. C. were the same, 99° to 101° C., which was about 55° C. lower than that reported by La

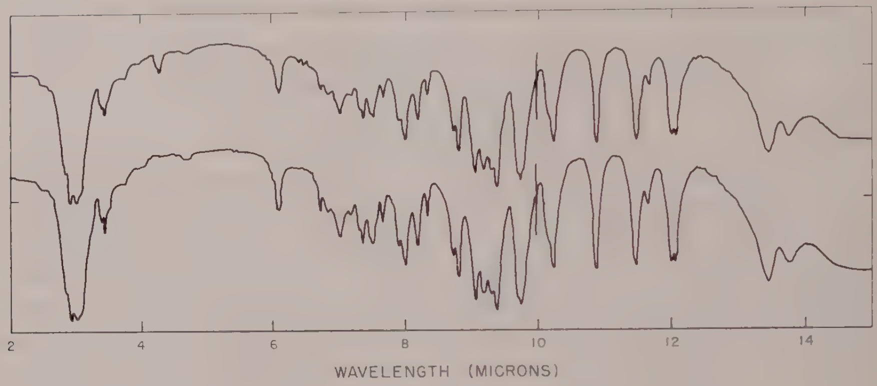

FIGURE 1. The infrared spectra of an authentic sample of sedoheptulosan (upper curve) and the sedoheptulosan from extracts of *Coleus Blumei* (lower curve).

Forge and Hudson (2). The discrepancy in melting points between the sample and that reported in the literature was probably caused by water of hydration. After both samples had been heated in an Abderhalden dryer for 4 hrs. at 100° C., they melted at 151° to 155° C. (uncorrected). The melting point of a mixture of the crystals from coleus and the authentic sample was 151° to 152° C. (uncorrected).

The infrared spectra and melting points of the white crystalline material obtained from *Coleus Blumei* are identical with authentic samples of sedoheptulosan. These data confirm the chromatographic evidence of Tolbert *et al.* (7) that sedoheptulose is one of the predominant free sugars of *Coleus Blumei.*

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3. NORDAL, A., and A. A. BENSON. Isolation of mannoheptulose and identification of its phosphate in avocado leaves. J. Am. Chem. Soc. **76:** 5054–5055. 1954.
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7. TOLBERT, N. E., C. W. NYSTROM, and P. C. KERR. Sedoheptulose in *Coleus*. Plant Physiol. **32:** 269–274. 1957.

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THE INFLUENCE OF WATER SUPPLY ON OLEORESIN EXUDATION PRESSURE AND RESISTANCE TO BARK BEETLE ATTACK IN PINUS PONDEROSA

J. P. Vité[1]

SUMMARY

The infestation of *Pinus ponderosa* Laws. by *Ips confusus* (Lec.) and *Dendroctonus brevicomis* Lec. is correlated with conditions of water stress in the tree. The seasonal and diurnal cycle of water supply leads to comparable cycles in the phloem moisture content and oleoresin exudation pressure.

Phloem moisture decreased with increasing height in the tree, was somewhat lower on the north side of most trees than on the south side, reached a low ebb some time after noon and had a tendency to increase from spring to fall. Changes in phloem moisture content were not directly correlated with success of bark beetle attack. Increases in moisture content apparently created a more favorable, rather than a restrictive, influence on the larval development.

Oleoresin exudation pressure (o.e.p.) was measured by inserting standard hydrostatic pressure gauges (of the Bourdon type) into holes bored into the sapwood. The gauges were lined with plastic or charged with glycerol to reduce their volume and to delay plugging of the orifice by resin. The pressure varied from 0 to 12 atmospheres depending upon site, weather conditions, soil moisture, and physiological condition of the tree.

O.e.p. change was directly related to the water stress in the tree. There was a regular diurnal cycle that reached a minimum of 4 to 8 atmospheres usually at 2 to 4 p.m. and a maximum of 8 to 12 atmospheres that prevailed between midnight and dawn but was appreciably modified by those climatic factors that influence the rate of transpiration such as temperature, light intensity and humidity. A declining o.e.p. was reversed within less than five minutes by sprinkling the atmosphere in the vicinity of the crown of the tree and within 20 minutes by reduction in light intensity by a cloud. The seasonal cycle of o.e.p. is primarily influenced by soil moisture and shows a gradual decline as the season progresses and available soil moisture is depleted. The minimum oleoresin exudation pressures, therefore, may be anticipated on hot, dry afternoons later in the season and are especially noticeable on trees growing in shallow soil on exposed sites. The ability of trees to restore normal o.e.p. during the night depends upon the water-procuring ability of the roots. Trees with a maximum o.e.p. of 4 to 6.5 atmospheres or less are in the minority and seem to be in subnormal physiological condition.

The success of bark beetle attack is closely correlated with low oleoresin exudation pressure. Initial attack occurs at random but only beetles in trees with low o.e.p. make a successful establishment that leads to a mass attack about 72 hours later. When the pressure in felled trees was prevented from dropping to zero by removing branches or pumping water into the logs the beetles were unable to colonize successfully the bark as long as some pressure remained. Also, trees under drastic surgery such as complete defoliation or interruptions of most of the sapwood resisted beetle attack as long as sufficient connection with the water supplying root system kept the o.e.p. above zero. Generally, resistance apparently

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prevails at o.e.p. above 4 to 6.5 atmospheres while trees with o.e.p. less than 4.0 atmospheres are susceptible to both bark beetle species investigated.

The correlation between o.e.p. and bark beetle infestation seems reliable enough to provide a sound basis for predicting the ability of individual trees to resist infestation. Such a method might be valuable in selective logging operations in second growth stands of ponderosa pine, especially on the western slope of the Sierra Nevada where other risk-rating systems have not proved reliable for removing trees susceptible to bark beetle infestation.

INTRODUCTION

The resistance of *Pinus ponderosa* Laws. to bark beetle infestation is obviously physiological in nature; inherent differences in resistance have not been described. Any tree resistant to infestation may become susceptible when subjected to drastic injury or change in environment. As a general rule, trees are predisposed to infestation by changes in water relations. Epidemics of subcortical feeding insects have long been associated with deficient rainfall or extreme droughts (3, 6, 9, 11, 13, 15, 16). Beetle infestations occur more frequently and are more destructive in stands on dry or exposed sites and where trees are exposed to comparatively high temperature and low humidity such as prevail at lower elevations (24, 35, 37).

The disturbances in water relations lead to changes in the moisture content[2] (37) and the turgidity of the phloem which could be measured by changes in osmotic pressures of the sap pressed from the phloem (17, 18, 24, 45). Among recent workers, especially Merker (24) and Zwölfer (45) were able to contribute considerable material showing the dependency of the resistance of forest trees to attack by subcortical feeding insects on water relations. Employing the kryoscopic method, however, it was not possible to establish critical values for the osmotic pressure prevailing in susceptible and resistant trees of the same species (45). Also, the gravimetric method of phloem moisture determination was bound to fail (24, 37, 43).

On the other hand, the oleoresin was recognized widely as an important factor in the resistance of conifers to bark beetle attack[3] (37, pp. 79–80). Systematic investigations on this subject, however, are rare and more recent. They are restricted to empiric measurements of oleoresin exudation from artificial wounds at the main stem of trees which had proven resistant or susceptible to actual bark beetle infestation (34, 37, 38) or were merely assumed to be resistant or susceptible by classification from the exterior appearance[3] (32). As is to be expected, individual trees form-ing large pitch tubes upon attack[3] or trees with heavy oleoresin flow (37, 38) and continuous resin exudation rated superior in resistance[3].

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[2] BEAL, J. A. Phloem moisture and beetle susceptibility of western yellow pine. Ms. report. Portland, Oregon. 5 pp. 1930.

[3] CALLAHAM, R. Z. Oleoresin production in the resistance of ponderosa pine to bark beetles. U. S. For. Serv. Ms. report, pp. 4–6. Berkeley, Calif. FS 2-11-11. 84 pp. 1955.

The idea, basic to the study presented, derives from both observations mentioned above and proposes that water stress causes susceptibility by reducing the oleoresin exudation. This hypothesis has been postulated by von Tubeuf (39, 40) and was advanced through the classical experiment by Münch (29) who showed that oleoresin exudation depends on the turgidity of a tree, i.e., the osmotic pressure in the epithelial cells lining the resin ducts. Some of the material of the recent investigations of Schwerdtfeger on *Ips typographus* L. infesting Norway spruce in Europe (37) and *Dendroctonus* species attacking pines in Guatemala (38) supports the same hypothesis. The relationship between the water relations, oleoresin exudation, and bark beetle infestation, however, remained to be proven experimentally.

Because of this, studies were initiated on the interrelationship between water supply, diffusion pressure deficit, phloem moisture, oleoresin exudation pressure, and severity of bark beetle attack on ponderosa pine growing on the western slope of the Sierra Nevada range in California. Data are presented in this report on the severity of attack and brood development of *Ips confusus* (Lec.) and *Dendroctonus brevicomis* Lec. under natural and artificially regulated conditions of water stress in ponderosa pine. In recognition of the importance of population dynamics (37), field observations were initiated under endemic as well as under epidemic conditions, the results of which will be reported separately (44).

Unfortunately, there are inadequate basic data on the various aspects of tree physiology and their relative importance in influencing and indicating resistance and susceptibility of conifers to bark beetle infestations. In addition, many of the conventional techniques of plant physiology fail when applied to tall trees, and considerable effort had to be spent on the search for suitable methods. In this attempt, the recent approach of Bourdeau and Schopmeyer (5) to measure oleoresin exudation pressure became an important basis for the development of a new technique.

MATERIALS AND METHODS

Observations and experiments were made in the Boyce Thompson Institute experimental forest at Grass Valley, California, where there is a second growth stand of ponderosa pine at an elevation of 2,200 to 2,500 feet on the western slope of the Sierra Nevada mountain range. Additional observations were made in the forests on the New Verde Mines Co. property at elevations of 3,600 to 5,200 feet and in the plantations of the Institute of Forest Genetics at Placerville, California. Trees of normal growth and appearance were chosen from typical sites except as indicated otherwise.

Measurements on water movement in trees were made as described previously (41, 42) by use of Acid Fuchsin or other dyes. Trees were denuded of branches either by cutting them off at the main trunk or at some distance from the trunk (Fig. 1 A). In some trees, the rate of transpiration was reduced by increasing the humidity in the crown region by means of a sprinkling system (Fig. 1 C). Water absorption was experimentally controlled by water injection into cut trees or by suspending a tree between two neighbors in its original position and feeding water to the cut off base (Fig. 1 D). Moisture content of the several tissues was determined by collecting samples of needles, phloem, sapwood, or heartwood early in the morning immediately upon felling the tree. The tissues were placed in a closed vessel of known weight, weighed and dried to constant weight at 102° C. Data are expressed as percentage of dry weight and calculations were made from these data on actual moisture content in various organs of the tree from their green weights. The water-holding capacity of a tissue at saturation was determined by immersing weighed samples from the freshly cut tree in distilled water for 24 hours and reweighing.

Phloem moisture content was determined by a modification of the method used by Hall[4] and Struble[5]. If not indicated otherwise, samples always were taken early in the morning from the north side of the tree by means of an arch punch 3.0 cm. in diameter which was driven through the bark into the outer sapwood. Preliminary trials had revealed that there was less statistical variation from samples of this size than those below 2.5 cm. or more than 5.0 cm. in diameter. The xylem and bark layers were carefully removed. The phloem was placed immediately in tared glass vessels, weighed and dried to constant weight.

Usually no difficulty was experienced in separating the phloem from the xylem but the separation of bark and phloem was somewhat more difficult. There is a moisture gradient within the phloem that decreases from the cambium toward the outer phloem so loss of the outer phloem may create a severe error in determining moisture. In order to keep such variations at nominal levels, it was found that 9 samples had to be taken to give a standard deviation of 5 per cent. The major disadvantage of the method employed was that in sequential tests it was impossible to measure the phloem in the same spot and mutilation during previous sampling undoubtedly had some influence on moisture retention by the phloem. Where climbing was necessary to obtain phloem samples, the Swiss Tree Climbers were used which allowed free movement and work along the bare bole without injury to the bark (Fig. 1 B).

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[4] HALL, R. C. An ecological study of *Ips confusus* in northern California. Ms. report. Berkeley, Calif. 22 pp. 1949.

[5] STRUBLE, G. R. A study of factors in the development of *Ips* outbreaks in ponderosa pine. Ms. report. Berkeley, Calif. 21 pp. 1949.

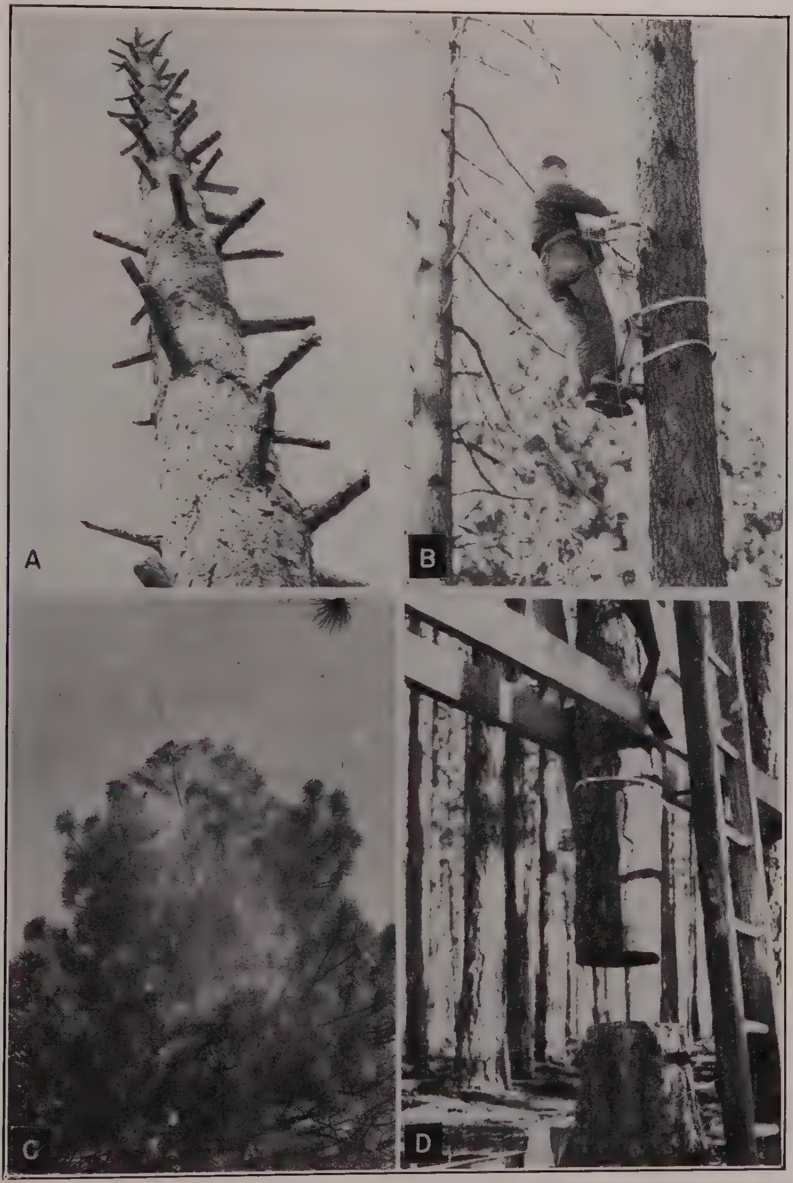

FIGURE 1. Techniques and tools used in the experimentation. (A) Defoliated ponderosa pine, branches were left to ease climbing; (B) Sampling phloem moisture and bark beetle infestation by means of the "Swiss Tree Climber"; (C) Sprinkling system attached to the top of pine was used to reduce transpiration; (D) Base of a pine suspended in its original position between two neighboring trees to measure and regulate water absorption.

The oleoresin exudation pressure was measured by two methods. After considerable experimentation based on the literature available (23, 26, 30, 34), it was found that the standard hydrostatic pressure gauge of the Bourdon type calibrated for pressures of 10 to 200 pounds per square inch (p.s.i.) could be used (Fig. 2). This method was chosen as being more simple and direct than Münch's (29) method of measuring the osmotic pressure of the epithelial cells that line the resin ducts or the use of mercury manometers (4, 19, 20, 21, 27, 31). The gauges used were accurate to 0.1 atmosphere. In some experiments instruments employing spiral and helical gauges with an accuracy of 0.05 atmosphere were used.

Three sources of error are inherent in the use of pressure gauges. The acids of oleoresin tend to crystallize sooner or later depending on its specific and individual consistency sealing off the resin ducts and clogging the instruments. Furthermore, the large variation in number and size of resin ducts within various portions of a tree, as well as from tree to tree, influences the volume of oleoresin available in each measurement. This, in turn, influences the time necessary to reach equilibrium between instrument and tree. Finally, the procedure of tightening the instruments to the tree requires a minimum of sapwood; trees with a layer of sapwood less than 5 cm. in thickness cannot be used.

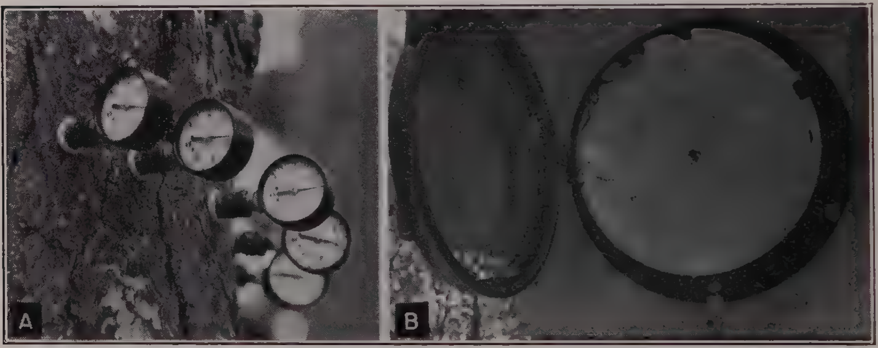

FIGURE 2. Instruments used in measurement of oleoresin exudation pressure. (A) Hydrostatic pressure gauges of the Bourdon type in position on a ponderosa pine. A 1-1/2″ metal nipple of 1/8″ pipe fastened to the gauge by a bell reducer is screwed into a hole of 3/32″ diameter and 2″ length drilled into the sapwood. (B) Recording gauge connected to the metal nipple screwed into the tree by means of a pressure tube filled with glycerol.

Attempts to prevent crystallization by use of a solvent such as xylene, or by discarding the oleoresin exuding first which was known to crystallize more rapidly[6], did not prove successful. Therefore, the crystallization of resin presents a special difficulty in trees with low rates of exudation requiring 24 hours or more to bring the gauges to full equilibrium. This was surmounted by reducing most of the volume of the instruments to capillary size by lining the metal nipples and bell reducers with plastic tubing or by filling them with a liquid such as glycerol prior to attaching to the tree.

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[6] SMITH, R. H. Resistance of pines to bark beetles. Studies on toxicity of resins. Ms. report. Berkeley, Calif. FS 2-11-11. 43 pp. 1959.

The duration of effective operation of the gauges varied widely in various tree species (43) and in different trees. In ponderosa pine, as a general rule pressure gauges were replaced every 24 hours but occasionally obstruction occurred during the first 24 hours. The consistency of the oleoresin varied from tree to tree so some trees showed a regular diurnal cycle for a period of four days, while on most trees the gauges worked properly for a day or two before a steady decline in pressure indicated a progressive obstruction of ducts and instrument. In order to avoid loss of data during the period while gauges were being changed, it was advisable to insert two gauges in those trees where records were to be taken over protracted periods of time.

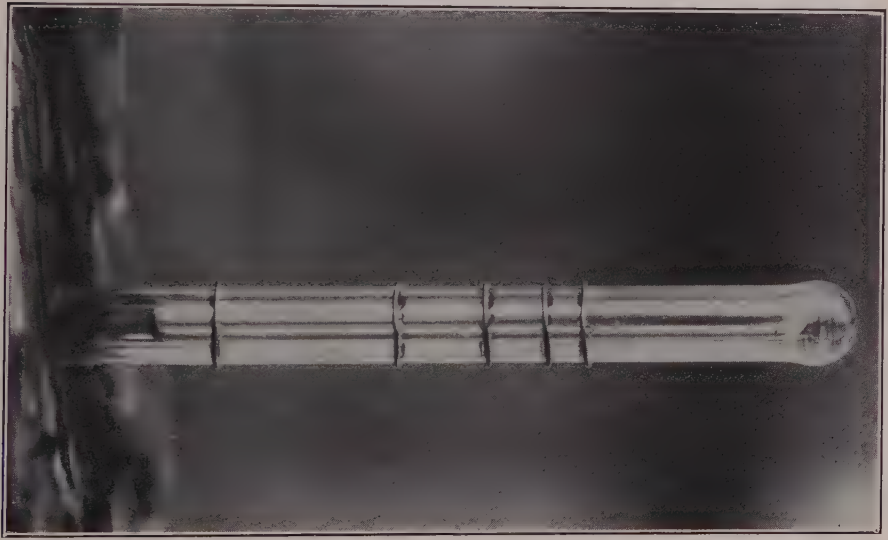

FIGURE 3. Measuring the oleoresin exudation pressure by means of a capillary glass tube.

The pressure gauges calibrated up to 200 p.s.i. were accurate only down to 10 p.s.i. so lower pressures were measured with low pressure gauges or estimated by a method similar to that developed by Bourdeau and Schopmeyer (5). Capillary glass tubes sealed on one end were partially filled with glycerol containing a red dye so as to entrap a column of air. The amount of compression of the entrapped air at different pressures in the range 0 to 60 p.s.i. was calibrated at 20° C. and the tube was appropriately marked (Fig. 3). The approximate o.e.p. could be determined by direct observation of the height of the red glycerol after the tube had been driven into a slightly narrower hole bored into bark and outer sapwood.

Generally, the instruments were read at intervals of two hours. Continuous readings of pressure at both low and high levels were accomplished by use of recording pressure gauges employing helical or spiral pressure elements (Fig. 2).

EXPERIMENTAL RESULTS

Water Movement and Moisture Content

As described previously (41, 42) water ascends in ponderosa pine in all sapwood layers of the tracheidal conduits. These deviate from a vertical plane at varying rates with age so the pathway upward from a given locus is in a spiral arrangement as shown in Figure 4 A. This connects each branch of the crown with each root so water demand can be supplied by the most direct route possible. This was readily demonstrated by removing all branches but one on a young tree and inserting the bole into a dye bath. The intake of dye followed the path of least resistance, that is, the tracheidal conduits with direct transpiration water deficit (Fig. 4 B).

However, a partial cut through the sapwood does not interrupt the upward movement of water as shown by others (8, 12, 33). In the dye experiments made in Grass Valley a partial cut of all sapwood layers as shown in Figure 4 C or a cut through the outer sapwood on the whole circumference of the trunk (Fig. 4 D) did not prevent water transport although it had to be accomplished against higher resistance at the periphery of the cut. Comparable movement of radioactive tracers has been reported recently in *Pinus resinosa* Ait. (33). Therefore, attempts to exclude certain portions of the sapwood from water supply did not succeed. Even two cuts through the entire outer sapwood disconnecting the midbole in these layers from the root and crown did not interrupt the water movement or reduce the phloem moisture anywhere (Fig. 4 E). Similarly, removing the branches from the top of tall trees did not change the moisture content of the bare top bole (Fig. 4 F).

The moisture content of ponderosa pines varied greatly from tree to tree, fluctuated with the season, during the day and from day to day. Similar variations were recorded along and around the trunk. Only some of these variables showed a definite pattern: the moisture content of the xylem increased radially from the inner to the outer layers, and vertically, from the base toward the top. The moisture content of the branches is less in the lower crown than in the top, while the phloem moisture increases from the top toward the base, and the moisture in the needles decreases with age. The gravimetric determination of the distribution of moisture in

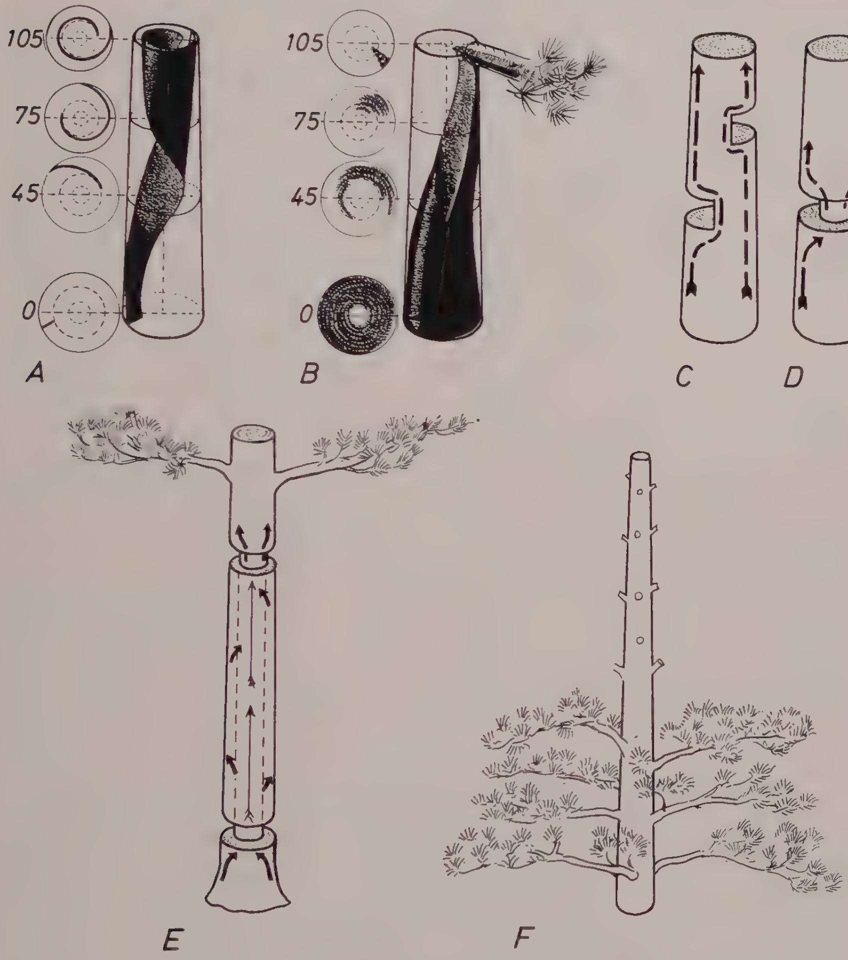

FIGURE 4. Ascent of moisture in ponderosa pine under normal conditions (A) and under drastic surgery (B–F) as explained in the text. Numbers in A and B indicate the heights of cross sections in centimeters above the base.

a 65-year-old ponderosa pine 27 meters high and 48 cm. in diameter at breast height led to the results presented in Table I.

Besides the radial and vertical differences in moisture content, circolateral differences occurred which were less distinct and irregular in pattern. Relatively high moisture in the outer xylem was generally accompanied by higher moisture in the immediate phloem tissue. The moisture content as expressed in saturation deficit was determined for the same tree (Table II). No significant differences were obtained in various portions of the trunk, either in the sapwood or in the phloem. The distribu-

TABLE I

MOISTURE CONTENT OF DIFFERENT SECTORS OF A PONDEROSA PINE OF AVERAGE GROWTH AT 65 YEARS OF AGE ON FEBRUARY 14, 1959

Sector of tree	Median age of wood in years	Height above ground in meters	Moisture content (% of dry weight)									
			Phloem	Sapwood			Entire branch, median age in yrs.			Needles		
				Outer	Middle	Inner	1–20	21–30	>30	'58	'57	'56
Top	17	20.25–27.00	149.5	158	—	—	121	—	—	107	100	88
Midtop	34	13.50–20.25	162.0	147	142	—	112	106	96	104	98	88
Lower crown	49	6.75–13.50	187.5	151	148.5	—	100	92	87	107	99	89
Base	56	0.50– 6.75	218.5	133	131	122				—	—	—

tion of absolute water content and dry weight of this tree is given in Table III.

Generally, the phloem moisture measured at breast height was found to vary between 170 per cent and 220 per cent but rarely higher than 250 per cent or lower than 150 per cent. Within this range the phloem moisture appeared to be variable to a limited degree only. Occasionally some trees exhibited differences of more than 20 per cent moisture based on dry weight, while on the average both the fluctuations on subsequent days and on different exposures were inconsequential (Table IV). Wider variations occurred in the diurnal and seasonal run. During the growing season, the phloem moisture decreased at the base of a tree from the morning toward evening, indicating the increasing water stress by transpiration; during the winter, reverse fluctuations were noted. An example is given in Figure 5 which shows the average phloem moisture of a 76-year-old ponderosa pine on one day of each season and at three different heights measured in the morning, at noon, and in the afternoon. The moisture fluctuated between 10 and 30 per cent of the dry weight. Wider variations of 20 to 50 per cent were observed in the seasonal changes of the phloem moisture as measured at the base of mature ponderosa pines (Table V). The data are gathered from earlier reports[4,5] and from observations at Grass Valley.

TABLE II

MOISTURE CONTENT OF A 65-YEAR-OLD PONDEROSA PINE EXPRESSED AS PER CENT OF SATURATION

Sector of tree trunk	Moisture content (% of saturation)			
	Phloem	Sapwood		
		Outer	Middle	Inner
Top	55.5	85.7	—	—
Midtop	54.8	84.0	86.8	—
Lower crown	52.1	83.5	86.8	—
Base	54.1	86.7	85.6	85.7

TABLE III

ABSOLUTE MOISTURE CONTENT AND DRY WEIGHT OF A 65-YEAR-OLD PONDEROSA PINE CALCULATED FROM GREEN WEIGHT OF VARIOUS PORTIONS OF TRUNK AND NEEDLES

Sector weighed		Green weight in kilograms	Dry tissue in kilograms	Total moisture in liters
Location	Part			
Top	Trunk	74	28.5	45.5
Top	Branches	104	40.2	63.8
Top	Needles 1958	20.3	9.8	10.5
	Needles 1957	12.4	6.2	6.2
Midtop	Trunk	286	116.4	169.6
Midtop	Branches	244	119	125
Midtop	Needles 1958	14.4	7	7.4
	Needles 1957	10.9	5.6	5.3
	Needles 1956	2.3	1.2	1.1
Lower crown	Trunk	511	204	307
	Branches	86	42	44
	Needles 1958	3.4	1.7	1.7
	Needles 1957	2.1	1.1	1.0
	Needles 1956	0.4	0.2	0.2
Base	Trunk	905	393	512
Roots (estimated)		300	120	180
Total		2,576.2	1,095.9	1,480.3

TABLE IV

EFFECT OF EXPOSURE ON THE PHLOEM MOISTURE CONTENT OF FOUR 95-YEAR-OLD PONDEROSA PINES

Sample used		Moisture content (% of dry weight and standard deviations)		
Tree No.	Side of tree	March 24, 1959	March 25, 1959	March 26, 1959
A	North	219 ± 2	212 ± 5	233 ± 1
	South	206 ± 1	207 ± 1	209 ± 1
B	North	196 ± 2	193 ± 5	178 ± 2
	South	197 ± 2	178 ± 2	174 ± 2
C	North	174 ± 2	176 ± 9	188 ± 3
	South	187 ± 1	182 ± 2	196 ± 1
D	North	193 ± 3	186 ± 4	190 ± 5
	South	194 ± 6	198 ± 5	196 ± 8
Average	North	195.5	191.8	197.3
	South	196.0	191.3	193.7
Average	North & south	195.7	191.5	195.5

There is some indication that the phloem moisture tends to increase from spring to fall as has been found for the sapwood moisture of other pines (10). However, site conditions and weather, physiological and phenological phenomena, such as the formation of foliage and needle drop, are expected to influence the seasonal trend of phloem moisture so strongly that it does not seem possible to establish any pattern from the material available. The most definite pattern and the largest differences in the distribution of phloem moisture throughout the year occur vertically along the trunk. From a sample containing 32 ponderosa pines, 80 to 160 feet in height, Hall[4] reported an average phloem moisture of 213 per cent at the base, 163 per cent at midtop, and 128 per cent at the extreme top.

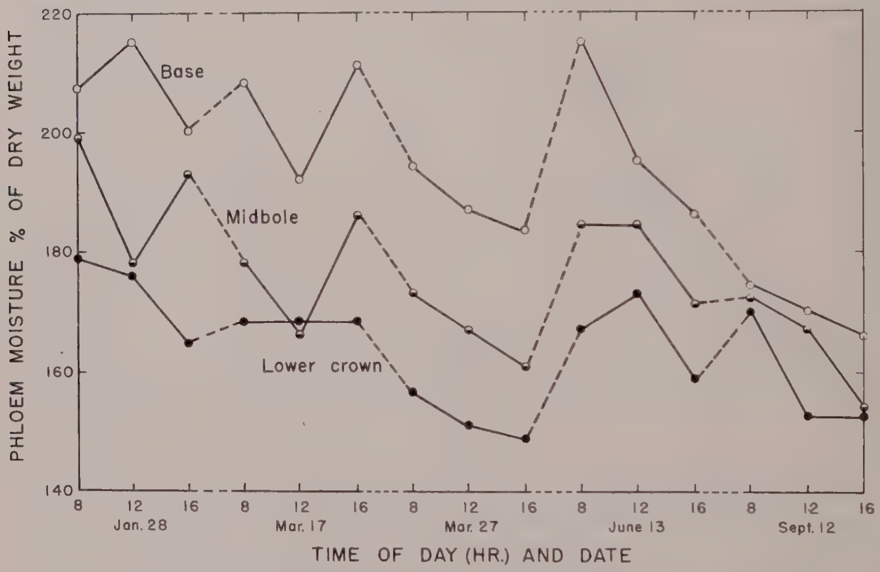

FIGURE 5. Diurnal fluctuations of phloem moisture at the base, midbole, and lower crown, of a 76-year-old ponderosa pine on five different dates in 1959.

Oleoresin Exudation Pressure (O.E.P.) in Pinus ponderosa

The close correlation between the water balance and the oleoresin exudation pressure (o.e.p.) was expressed in a distinct diurnal fluctuation depending on site and weather conditions influencing water absorption and moisture loss by transpiration. While the latter was found responsible for the diurnal run of the o.e.p., the water absorption through the root system and accordingly the soil moisture determined the pressure level.

The diurnal fluctuation. The diurnal fluctuation of the o.e.p. followed directly the run of transpiration (Figs. 6, 7, 8, 9, 10, 11). With the highest

TABLE V

SEASONAL VARIATION OF PHLOEM MOISTURE IN PER CENT OF DRY WEIGHT AT THE BASE OF MATURE PONDEROSA PINES; RANGE IN PARENTHESES

Observer	Year	Location	No. of trees	March	April	May	June	July	August	September	December
Hall*	1947	Northern California	12	—	—	—	—	210	177	165	—
	1948		15	—	199	185	201	197	230	233	—
Struble*	1948	Southern Cal. (dry site)	5	—	—	—	157.7 (140–203)	183.2 (155–209)	—	184.0 (166–218)	—
	1948	(moist site)	5	—	—	—	216.4 (194–233)	194.1 (186–202)	—	185.1 (178–195)	—
Vité	1958	Grass Valley	7	179.1 (159–197)	—	—	211.6 (182–236)	—	—	205.6 (172–238)	185.6 (166–211)

* See footnotes [4,5] in text.

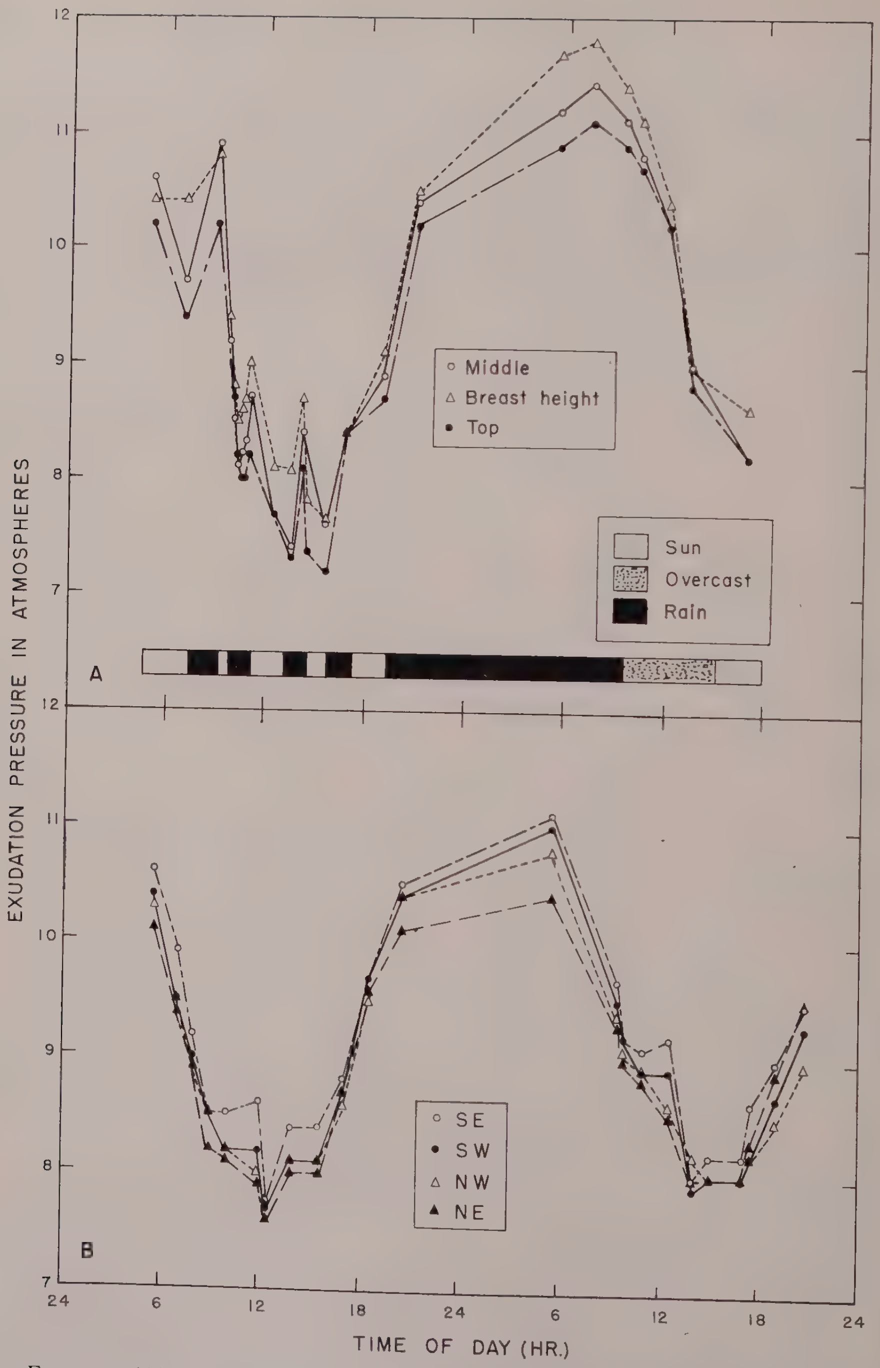

FIGURE 6. (A) Vertical differences in the o.e.p. in a 63-year-old ponderosa pine during a period of changing weather conditions, May 22–23, 1959. (B) Circolateral differences in the o.e.p. in an 80-year-old ponderosa pine, May 8–9, 1959.

value at dawn, the pressure dropped as the diffusion pressure deficit increased. The lowest pressure was found in the early afternoon during the spring and in the late afternoon during the summer. With the decline of the transpiration, the o.e.p. increased in the evening and during the night. Trees with the crown exposed to the east as well as stands exposed to the same direction showed a faster decrease of the o.e.p. in the morning than trees exposed to the west.

Pressure variations within a tree. Within a tree, the o.e.p. may vary around and along the trunk. Such variations were more distinct at the high pressure level during the night and in the morning than under water stress during the daytime. Usually, the pressure was somewhat higher in the lower bole than in the midbole and the top. Circolaterally, differences were obtained but they were individual in character and not predictable (Fig. 6).

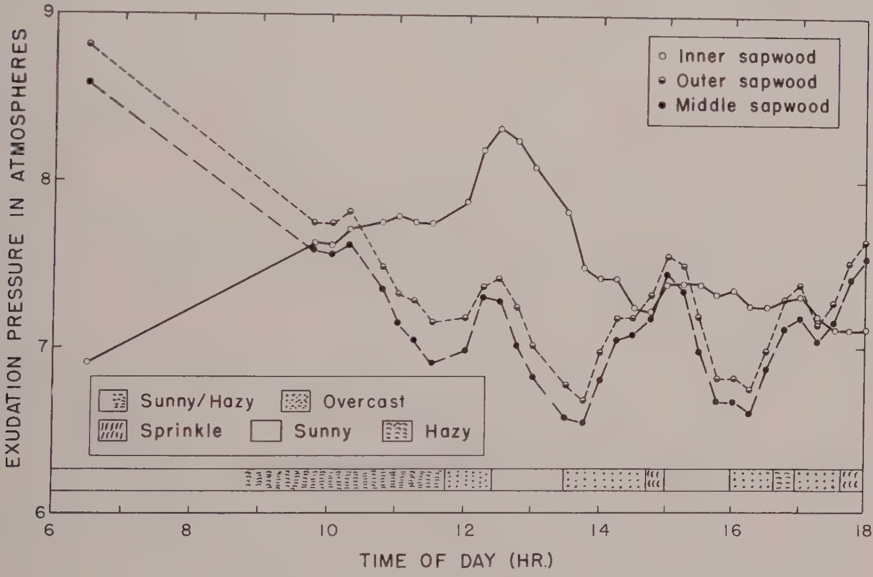

FIGURE 7. Radial differences in o.e.p. in a 95-year-old ponderosa pine, May 24, 1959.

Major differences occurred radially. The o.e.p. was found to be lower in the inner sapwood where changes due to the transpiration were retarded and moderate. Figure 7 presents the o.e.p. measured in different sapwood layers of a 95-year-old ponderosa pine during a day of changing weather conditions. During the morning hours, the o.e.p. increased in the inner sapwood while it was dropping rapidly in the outer sapwood. Later in the day, clouds and rain caused temporary increases of the pressure in the outer sapwood while the inner sapwood did not seem to be affected.

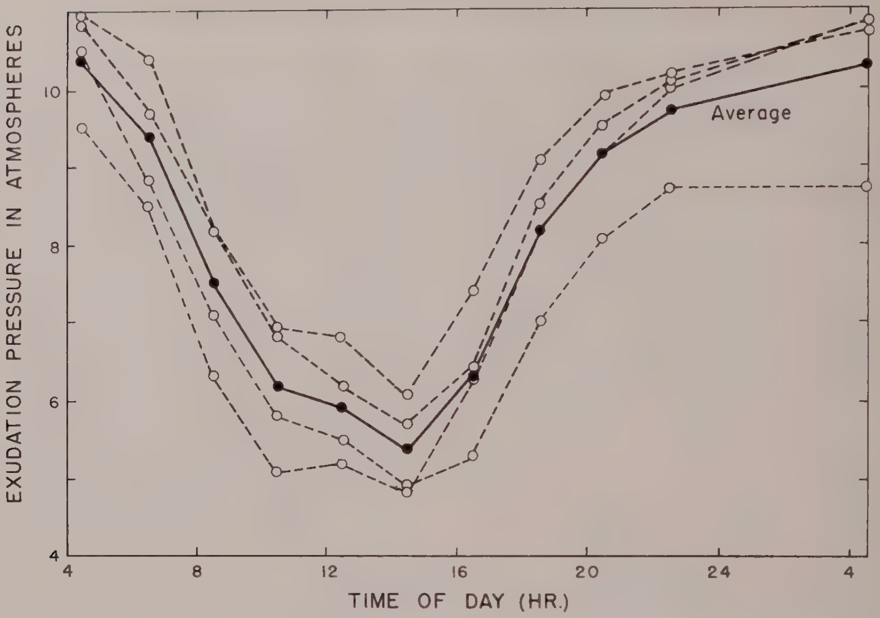

FIGURE 8. Individual differences in o.e.p. in four 90-year-old dominant ponderosa pines of similar growth rate and crown shape, June 24–25, 1959.

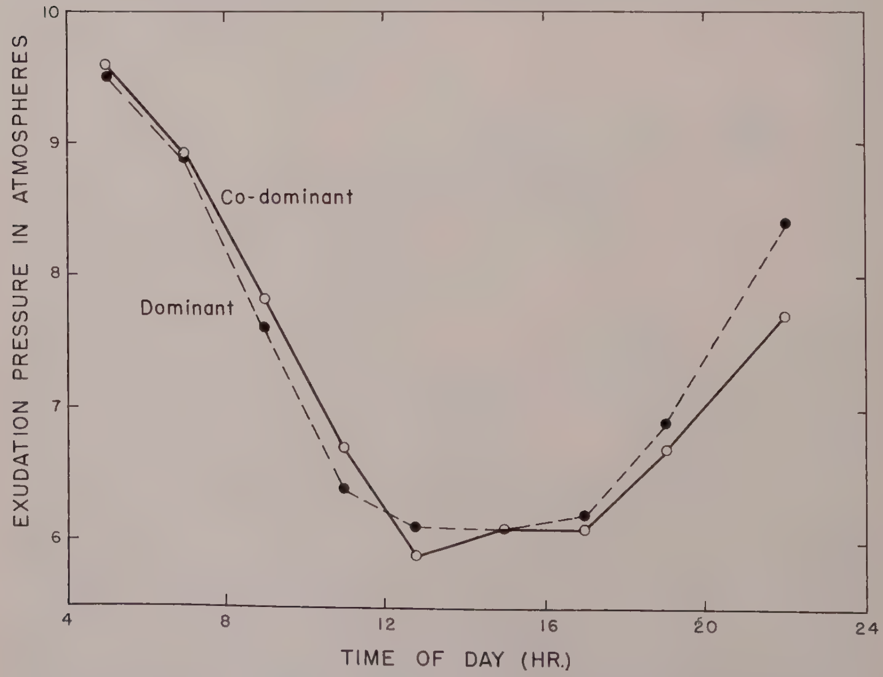

FIGURE 9. Average oleoresin exudation pressure of 6 dominant and 6 co-dominant ponderosa pines of the same stand throughout a day.

Pressure variations from tree to tree. Under comparable conditions, i.e., at the same time and location, the o.e.p. varies only moderately from tree to tree (Fig. 8). At dawn and on sites with soil moisture close to field capacity the o.e.p. ranged in ponderosa pines between 9 and 11 atmospheres. Occasionally higher pressures of 12 to 13 atmospheres occurred after a period of rain with the humidity close to 100 per cent. The absolute maximum observed under those conditions was close to 13.7 atmospheres in a single tree, at dawn. Trees with o.e.p. lower than 8.0 atmospheres in the morning were the exception in vigorous second growth stands, and those with o.e.p. less than 6.5 atmospheres were in the minority on exposed sites. Trees with relatively low o.e.p. in the morning exhibited a low level of o.e.p. throughout the day. Except for overmature trees, this appearance was definitely not correlated with age or tree class (Fig. 9). Ponderosa pines 30 to 240 years of age as well as dominant, intermediate or suppressed trees did not exhibit a significantly different o.e.p. level.

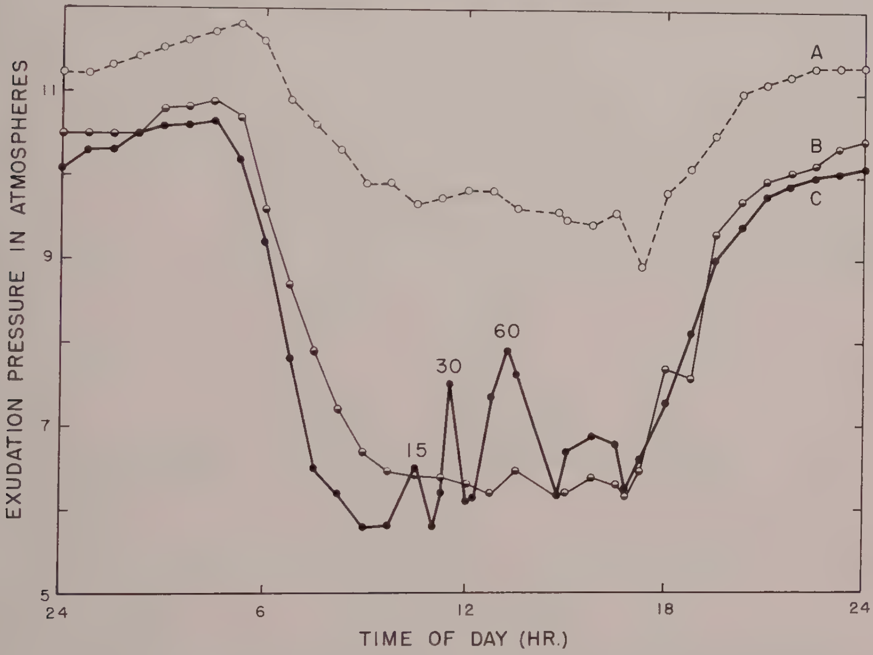

FIGURE 10. The o.e.p. in an 80-year-old ponderosa pine due to experimentally changed humidity: (A) clear weather, permanent sprinkling; (B) clear weather; (C) clear weather, temporary sprinkling of the crown for 15, 30, and 60 minutes.

Variations due to weather. Light, low humidity, and wind which increased the moisture loss by transpiration, decreased the o.e.p. while clouds on a sunny day caused temporary increases in o.e.p. (Figs. 6, 7).

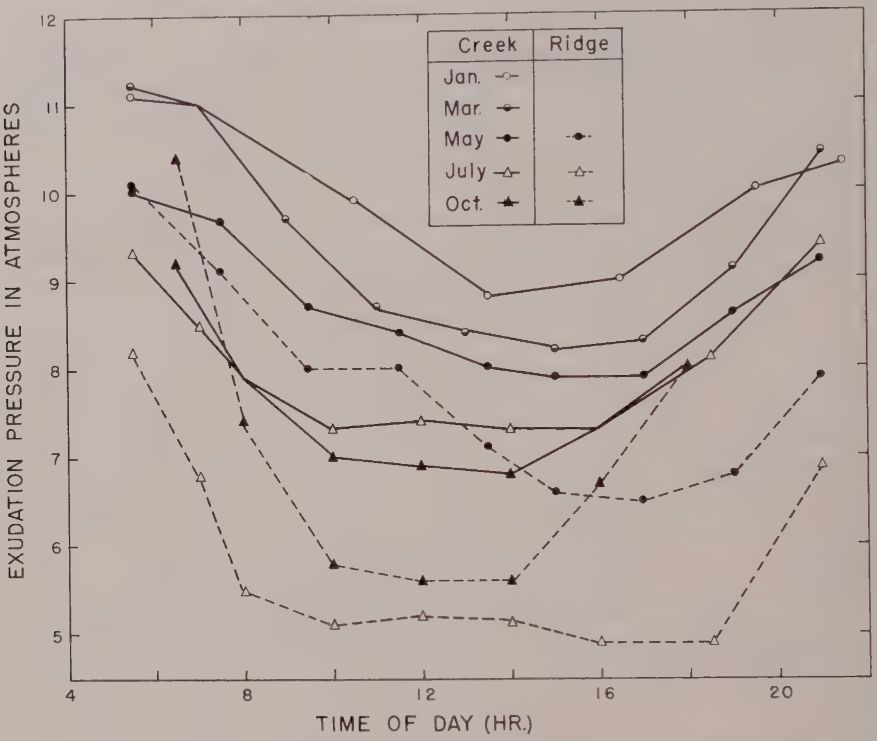

FIGURE 11. Seasonal variations in o.e.p. in 90- to 100-year-old ponderosa pines on a moist (Creek) and a dry (Ridge) site of the same elevation.

The shade of clouds became effective within 15 to 20 minutes in tall, 60- and 95-year-old ponderosa pines increasing the o.e.p. from 0.5 to 1 atmosphere.

To demonstrate the influence of humidity on the o.e.p. experimentally, a ponderosa pine 97 feet in height and 80 years of age was covered with a sprinkling system and the influence of sprinkling on the o.e.p. observed by means of a recording pressure gauge. Permanent sprinkling reduced the diurnal fluctuation of the o.e.p. to a minimum; the pressure dropped only 2 to 3 atmospheres where a decrease of 5 atmospheres would have occurred normally. Sprinkling during the daytime for 15 minutes increased the o.e.p. 0.7 atmosphere; 30 and 60 minutes sprinkling resulted in an increase up to 1.8 atmospheres for the respective periods of time (Fig. 10). In addition, continuous sprinkling raised the soil moisture to field capacity which resulted in an increased o.e.p. of about 1 atmosphere.

Seasonal variations in o.e.p. With the highest level in January, the o.e.p. decreased slowly with the increasing transpiration to March and

May and more rapidly throughout the summer and fall with the progressing deficit in soil moisture.

Experimentally, the level of the o.e.p. can be restored by watering. However, the effect of watering individual trees was not so immediate. Trees watered during the daytime for several hours did not show an immediate response in the o.e.p., but the pressure increased during the night to a maximum level. Water was contained around the roots of three ponderosa pines by metal collars approximately the size of the main root system. The o.e.p. showing an average of 9.5 atmospheres after 21 to 25 days of dry weather, was brought back within 24 hours to the original average of 10.4 atmospheres o.e.p. as measured previously after a rainy period.

Pressure variations due to site. Most obvious was the influence of transpiration and soil moisture on the o.e.p. of ponderosa pine stands on different sites. This influence found its expression in both the seasonal variation and the diurnal fluctuation. Ponderosa pine stands, 95 years old, on a dry site and a moist site in the immediate neighborhood were investigated throughout the year (Fig. 11). On the dry site the diurnal fluctuation was more extreme than on the moist site of the same altitude, due to

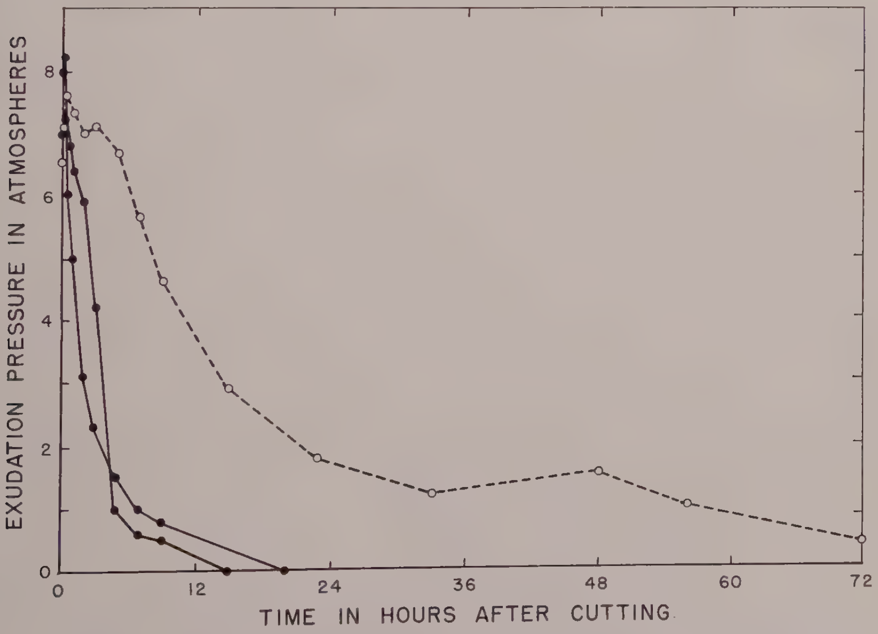

FIGURE 12. Loss of o.e.p. in two ponderosa pines with intact crown (solid line) and in a trimmed tree (broken line). Dec. 4–6, 1959.

the increased diffusion pressure deficit. On both sites the diurnal fluctuation became greater and the level of the o.e.p. lower as the season progressed and the soil moisture decreased.

Influence of Drastic Surgery on Bark Beetle Attack

Drastic interruptions of the phloem tissue and the water conducting xylem, as well as drastic environmental changes and water injections were applied to investigate whether trees or portions of trees could be physiologically predisposed to bark beetle attack.

Cutting. Complete removal of a tree from its stump reduced the o.e.p. within 24 hours to zero pressure, provided the branches were retained. The fast decrease of the o.e.p. is mainly due to the moisture loss by transpiration when the branches are left on the trunk. If the branches were removed immediately after felling the tree, the o.e.p. decreased more slowly and some pressure was retained for a period of several days (Fig. 12). It was repeatedly noted that immediately after the cut, the o.e.p. rose 0.2 atm. to 1.0 atm. This might be explained through the phenomenon of contraction of the water columns after tension is released by the cutting.

In contrast to the o.e.p., phloem moisture is known to change slowly in trimmed trees and in untrimmed trees where the moisture loss by transpiration is continuous. In trimmed ponderosa pines, the trend of the phloem moisture was upward, while in logs with branches the trend was downward. Under both conditions, phloem moisture was not found to be a limiting factor either in beetle attacks or in brood development[5].

Experiments confirmed these findings although they were undertaken under high temperature conditions during midsummer 1958. Even the trimmed tree sections showed a slight decrease in phloem moisture, while the phloem moisture in the stump remained at a normal level or increased somewhat. Injecting water under a pressure of 2 to 3 atm. through a hole bored into the trunk kept the phloem moisture in untrimmed trees on a level slightly above normal. In the early morning a sharp decrease during the daytime indicated a strong diurnal pattern according to the run of moisture loss by transpiration. In trimmed logs, water injections increased the phloem moisture steadily over a long period, eventually to the point of saturation. Figure 13 represents the data for the phloem moisture obtained from differently treated sections of the same ponderosa pine. All data refer to samples taken during the morning.

Under the prevailing high population of *Ips confusus* all felled trees were attacked readily by this species and occasionally by individuals of *Dendroctonus brevicomis*. Usually, the initial attack which was started by only a few beetles occurred within the first three days after the cut, but primarily during the second and third day and with preference for trees that had branches. This was followed by a mass attack during the next 2 to 3 days. All logs were attacked whether they were water injected, trimmed or not trimmed. However, in all 7 repetitions throughout the summer 1958, beetle attacks on the water injected logs which were trimmed, or branchless, failed. The beetles were found dead in short galleries incrusted with oleoresin or the galleries were abandoned before completion. Deposition of eggs and larval development were found occasionally only on the ends of the logs. As water was injected at the center of the log, it became obvious that water did not reach these areas. When the water injection was discontinued for about two weeks, *Ips confusus* broods then were able to develop. The water injections, however, often led to fermentation of the cambium, the "sour cambium condition" which in turn created unfavorable conditions for the brood development.

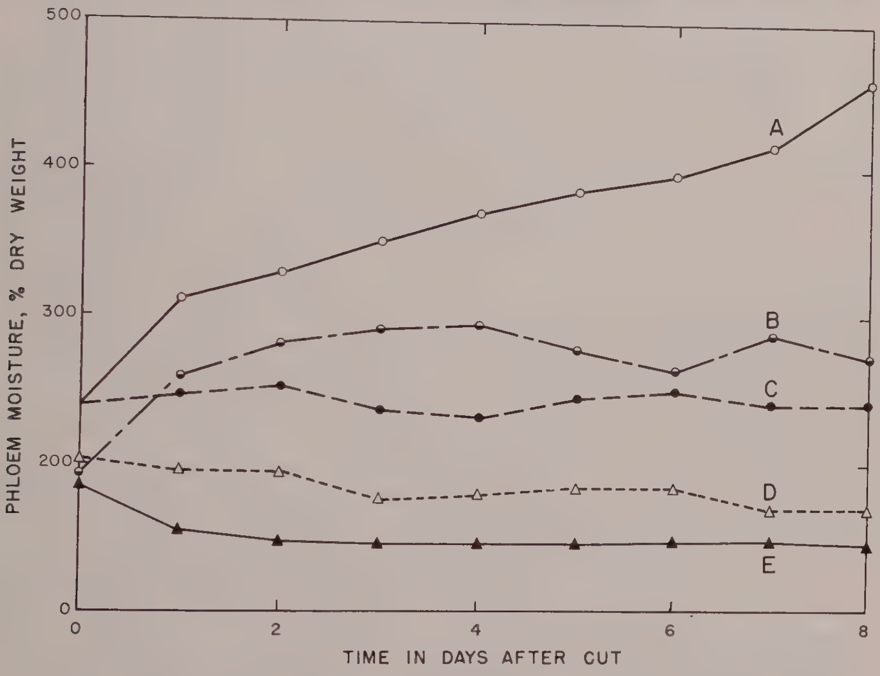

FIGURE 13. Phloem moisture in branchless water injected (A), intact water injected (B), tree sections and in the stump (C), branchless (D), intact (E) of the same ponderosa pine. Cut on July 23, 1958, 8 a.m.

Water injections alone did not suppress egg deposition and development of brood in logs that had branches. Here, the brood seemed to find rather optimal conditions despite slightly above normal phloem moisture. Similar observations were recently reported by Anderson (1) on *Ips pini* in Jack pine, *Pinus banksiana* Lamb. Brood production and survival were highest when the "inner bark" moisture did not deviate too greatly from that found in "vigorous trees", around 200 per cent moisture. Even phloem moisture as high as 250 to 300 per cent was still rather favorable (1, p. 601).

Girdling bark and sapwood cuts. Girdling the bark including phloem and cambium as well as deep cuts into the sapwood on the whole circumference of the trunk did not interrupt the water supply nor strongly influence the o.e.p. Also, there was no effect on bark beetle attack. Ponderosa pines treated in these ways remained resistant for one or more seasons, unless subsequently heavily exposed.

TABLE VI

EFFECT OF DRASTIC SURGERY ON INFESTATION BY BARK BEETLES AND FLATHEAD BORERS

Treatment	No. of trees	Attacked by beetles	Un-attacked	No. of trees	Attacked by beetles	Un-attacked
		Exposed to light and wind			Not exposed to light and wind	
Bark girdled on whole circumference	3	3	0	11	3*	8
Cut through bark and $\frac{1}{3}$–$\frac{2}{3}$ of sapwood on the whole circumference	3	3	0	11 (5 thrown by wind)	2*	4
Partial sapwood cut $\frac{3}{4}$ of circumference	1	—	—	1	1	—
$\frac{1}{2}$ " "	—	—	—	1	—	1
No treatment	4	0	4	15	1*	14

* Suppressed trees of small size.

An experiment consisting of a block of 49 ponderosa pines in which different treatments were applied in a Latin square arrangement was performed in early April, 1958. Circolateral interruptions of the bark below the crown or at breast height and circolateral cuts through the bark into the sapwood to a depth of one-third to two-thirds its diameter were made. Ten of these trees on the southern border of the plot were exposed to light and wind by removing the whole stand in front of it. At the end of the second season, in October 1959, 13 trees had died, of which one remained unattacked, while 12 trees were attacked by bark beetles and flathead borers. From a total of 19 untreated check trees only one small-sized tree was attacked and killed. Of the remaining 36 trees, 5 were lost from windfall and subsequently attacked by *Ips*, while the rest of the trees remained alive and unattacked. The results of this experiment are compiled in Table VI. Of the trees which had received drastic surgery, only those which had been exposed or were suppressed and small in size were attacked and killed.

In this experiment, *Dendroctonus brevicomis* occurred in the large diameter trees, while the 4 smaller suppressed trees were attacked by flathead borers either alone or together with *Ips*. From the trees attacked by bark beetles, 2 were attacked during the first season and 8 during the season of 1959. Five of those killed in 1959 possessed a sour cambium condition and little surviving brood was observed. The surgical treatments did not seem to influence the choice of host material by the beetle species involved. However, this conclusion is based only on a small number of observations.

In further experiments two deep cuts into the sapwood on the whole circumference of the trunk were made, one below the crown and one at breast height (Fig. 1 E), so that the whole outer sapwood of the lower bole was separated from the root system and the crown. However, this treatment did not sufficiently disturb the water supply and the o.e.p. to induce susceptibility to that portion of the tree which was vertically and circolaterally disconnected from the crown and the roots. Only when the cuts were so deep that more than two-thirds of the water conducting area was interrupted was any effect observed. Two neighboring trees were treated in this manner on March 1, 1959. At the end of May, the o.e.p. dropped to zero only in tree B where three-fourths of the sapwood layers had been severed. In early June this tree was attacked and later killed by *Ips* sp. and *Dendroctonus brevicomis*, while tree A remained unattacked but exhibited a somewhat reduced o.e.p. throughout the summer (around 8 atm.) and fall (around 6.5 atm.). In measuring the o.e.p. below and above the cut, it was noted that the o.e.p. below the cut was generally lower during the night but higher during the daytime showing more or less a reversed trend of the normal diurnal fluctuation.

Topping and debranching. Partial or complete loss of the green crown occurs frequently in ponderosa pine second growth due to snow or wind breakage or through breakage caused by disease. Generally, the felled tops are readily attacked by *Ips*, while the stumps remain unattacked for a longer period or, on many occasions, are not attacked at all. The latter is especially true where a large number of green limbs are left on the bole keeping the tree alive indefinitely.

To study the effect of a complete loss of the crown, 9 ponderosa pines were topped at 11 meters aboveground and all branches removed from the remaining stump at different times. While the cut off tops from 7 trees out of 9 were readily attacked by *Ips* within 5 days, the stumps remained unattacked for at least 23 days in 7 instances and for 42 days in another. In the 9th tree the stump was not attacked; this was probably due to the low bark beetle population level at the time and location of this repetition. In accordance with the observations of Anderson (1) on *Pinus banksiana*, the phloem moisture in these stumps increased to about 100 per cent above normal without affecting beetle attack or brood development. Again a close correlation was found between the o.e.p. and beetle attack. In one of the stumps the o.e.p. as well as the phloem moisture was measured. Here, beetle attack occurred a week after the o.e.p. at the base had dropped to zero.

A closer study of the o.e.p. in such treated trees showed that after debranching, the o.e.p. fluctuated irregularly throughout a day but increased somewhat after a temporary drop because of the surgical treatment. Later, the o.e.p. decreased slowly to zero, first in the lower bole, eventually in the top (Fig. 14). There were some indications that phloem moisture as well as o.e.p. depended upon the soil moisture; after rain higher values in phloem moisture were obtained. On moist sites and early in the year the o.e.p. dropped more slowly than on dry sites and during the summer.

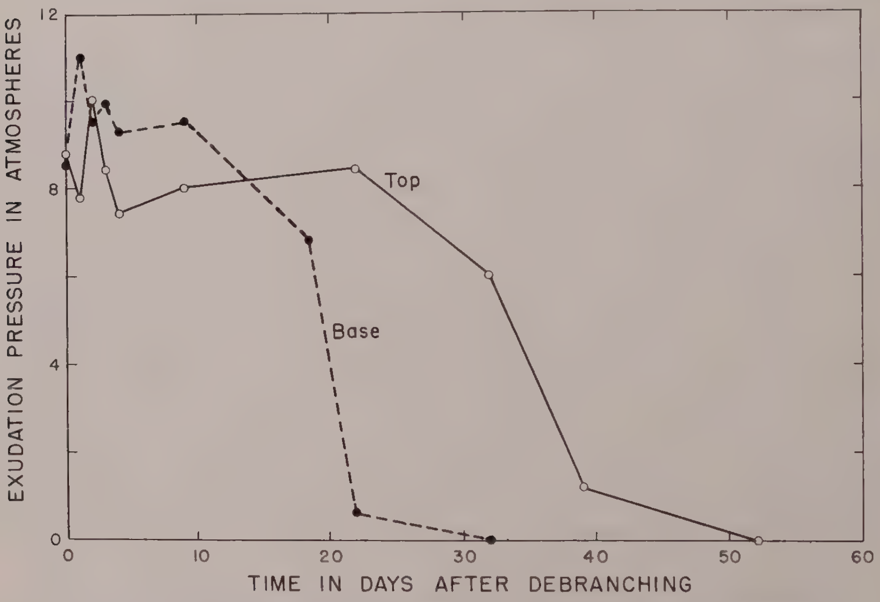

FIGURE 14. Decrease of the o.e.p. in the base and top of an 11-m ponderosa pine after removing all branches. June/July, 1959.

Partial debranching of trees did not change the phloem moisture significantly but increased the o.e.p. in two repetitions from 1 to 1.5 atm. Even cutting off all of the top branches did not influence phloem moisture in this particular portion of the tree. Six years ago, a 29 meter ponderosa pine, the top of which was debranched for 3 meters in order to install a TV antenna, possessed the same phloem moisture and o.e.p. in the bole both below and above the uppermost branches. Even trimming a large portion of the top bole did not change this situation (Fig. 15). That this phenome-

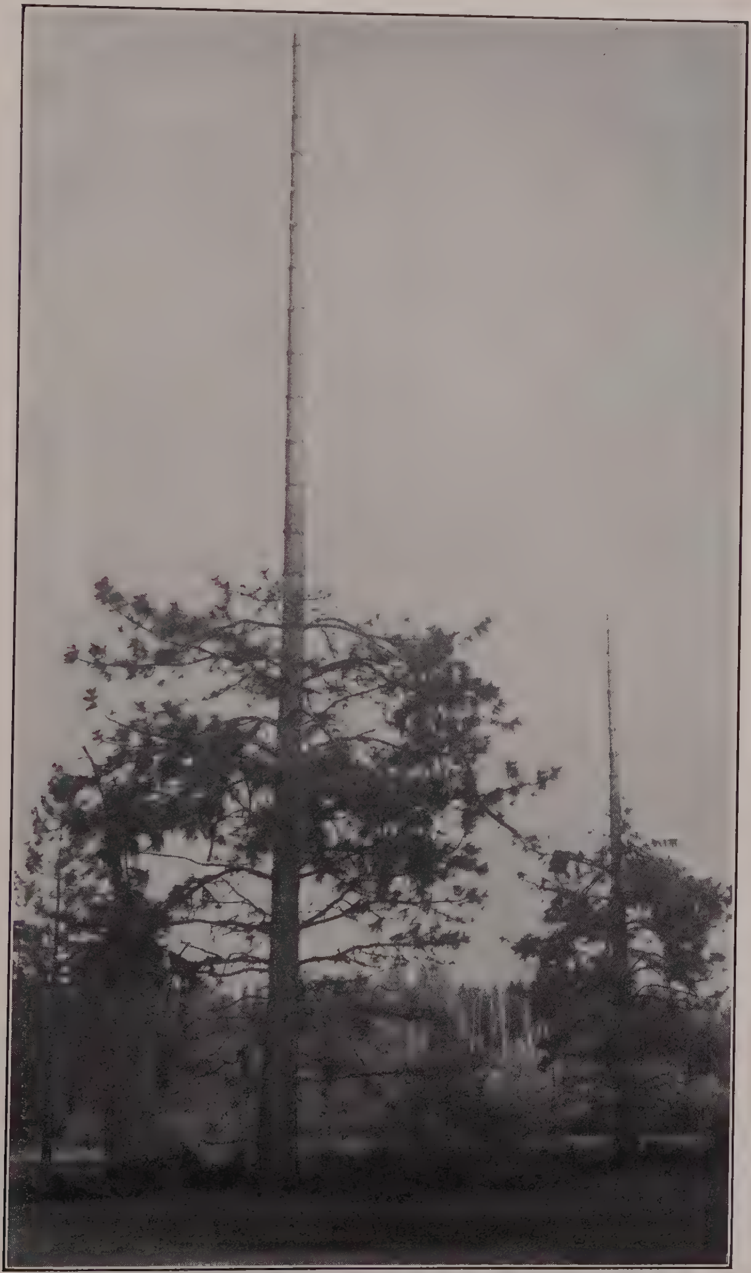

FIGURE 15. Partial removal of branches from a 60-year-old ponderosa pine.

non is the rule rather than the exception can be concluded from the fact that the tree tops of ponderosa pines killed by *Ips* break down in the first or second season, while trimmed tops last an indefinite time despite the weight of a TV antenna, e.g.

Exposure. The greatest influence on the resistance of ponderosa pines to bark beetle attack was achieved by drastic exposure of trees to light and wind by removal of sheltering neighbors. Out of 12 trees exposed to the southwest in March 1958, 3 trees were attacked and killed by *Dendroctonus brevicomis*, 1 by *Dendroctonus brevicomis* and *Ips* during the same season.

DISCUSSION

From the experiments described it is concluded that the predisposition of ponderosa pines to bark beetle infestation is due to disturbances in the water relations which decrease the turgidity considerably beyond diurnal, seasonal or local fluctuations. These disturbances may be of a temporary character due to sudden changes in the environment such as exposure through logging, fire, previous bark beetle attacks and droughts, or they may be of a more permanent nature through lethal damages or increasing unfavorable conditions such as competition. Temporary predisposition is reversible through adaptation of the root system and the transpiring surface to the new environmental conditions within one or more seasons. The slow process of regaining the normal physiological condition, however, exposes the tree for one season or more to bark beetle attack.

In other words, susceptibility to bark beetle attack occurs where transpiration and absorption are out of balance. Lack of transpiration water leads to a steadily increased diffusion pressure deficit throughout the tree and subsequently to a low turgidity in the living tissues. With decreasing turgidity in the epithelial cells lining the resin ducts, the rate of exudation in which the oleoresin exudes from the interconnected capillary system of resin ducts (22) diminishes (2, 5, 28, 39, 40). Oleoresin flow is a major factor influencing the success of the initial bark beetle attack (14, 32, 36, 38), while mass attack seems largely to depend upon whether or not the initial attack was successful. As an expression of both the water balance of the tree and its oleoresin flow, the oleoresin exudation pressure appears to be a dependable indicator of the physiological condition of a ponderosa pine and therefore its resistance or susceptibility to bark beetle infestation.

Although it was possible to demonstrate experimentally the immediate effect of the water relations on the o.e.p. and the close correlation between successful bark beetle attack and low o.e.p., it is not assumed that the resistance of ponderosa pine to bark beetle attack is exclusively physical in nature. Other mechanisms of resistance, especially the ability to produce oleoresin[3], and also to form traumatic tissue, and the secretion of secondary resin therefrom (18), may be similarly higher in trees with normal turgidity than in trees in a subnormal physiological state.

From investigations of the conduction and distribution of moisture, it can be concluded that a direct influence of the moisture *per se* on bark beetle attack does not exist. The state rather than the amount of water is indicative of the physiological condition of a plant; quantitative expressions of phloem or sapwood moisture based on dry weight percentage proved useless in the determination of tree susceptibility. The experiments, however, show that large portions of the sapwood probably serve in ponderosa pine as in other plants (25) as a water reservoir. Even the obstruction of a large portion of the water conducting cross section does not necessarily influence the water balance. The transpiration water normally transported in the outer growth rings can ascend equally well through the inner sapwood layers. Due to the diurnal and local fluctuations of the diffusion pressure deficit and the redistribution of water resulting from a normal distribution of moisture and turgidity the o.e.p. is maintained even in those portions of the main stem which have been separated from each other through deep circolateral cuts on the whole circumference. Therefore, it was not possible to render some portions of the tree artificially susceptible to bark beetles while leaving other sections of the tree resistant. This phenomenon indicates that a tree can normally tolerate severe wounds, for instance logging damage, without seriously affecting the over-all physiological condition.

In bark beetle research, the applicability of the findings and methods of measuring o.e.p. reported here, may lie in the preventive determination of the susceptibility of individual trees as well as stands of second growth ponderosa pine to bark beetle infestation. The results of Eaton's study (7) on bark beetle damages in ponderosa pine stands on the eastern range of the Sierra Nevada suggest "that once the susceptible trees are logged, sharp reductions in the volume of timber killed can be expected, regardless of the harvesting method." Accordingly, the method of o.e.p. determination might be helpful in the management of the ponderosa pine second growth on the western slopes of the Sierra Nevada Mountains where the conventionally used risk rating system does not apply.

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A STUDY ON THE APPLICABILITY OF THE MEASUREMENT OF OLEORESIN EXUDATION PRESSURE IN DETERMINING SUSCEPTIBILITY OF SECOND GROWTH PONDEROSA PINE TO BARK BEETLE INFESTATION

J. P. Vité[1] and David L. Wood[2,3]

SUMMARY

Earlier experimental observations on the relationship of oleoresin exudation pressure (o.e.p.) and tree resistance to bark beetle attack, as well as a technique of measuring o.e.p. by means of hydrostatic gauges, were subjected to field trials on various sites of second growth ponderosa pine at the north end of Lake Tahoe in Nevada. Trees were classified as to anticipated resistance according to their o.e.p. at the onset of heavy natural invasion by *Dendroctonus brevicomis* Lec. and *Dendroctonus monticolae* Hopk. during the summer of 1959 and 1960. A total of 424 and 544 ponderosa pines were observed in 1959 and 1960, respectively.

In 1959, 138 trees or 32.5 per cent were attacked, 98 of them unsuccessfully. Of the 40 trees attacked successfully 33 had an o.e.p. less than 4 atmospheres. Only one out of 34 trees with an o.e.p. less than 4 atmospheres survived the mass attack, while only 7 out of 24 trees which were mass attacked with o.e.p. greater than 4 atmospheres were killed. Due to extreme drought conditions and the high population level of beetles in 1960, a total of 212 trees or 39 per cent were attacked, 74 of them unsuccessfully. From 133 trees with o.e.p. less than 4 atmospheres, 104 succumbed but only 34 trees out of the 79 trees with o.e.p. greater than 4 atmospheres were overcome by a mass invasion.

Trees of both low and high o.e.p. were initially attacked at the same rate but subsequent mass attack occurred much more frequently in trees with low o.e.p. where the successful establishment of the initial attack appeared to create a mass attraction. Differences in susceptibility due to site and stand conditions among various plots were found to be expressed in the percentage of trees with high and low o.e.p. As a silvicultural practice to prevent bark beetle outbreaks, measurements of the o.e.p. are suggested for risk-rating individual trees as well as stands of second growth ponderosa pine.

INTRODUCTION

The long period involved in the production of forest crops constitutes many important problems, of which the prevention of losses by insects is one of the most critical. In coniferous forests, the annual toll taken by bark beetles not only represents substantial reductions of timber reserves and increment, but also serious disruptions of forest management planning. Therefore, the early recognition of potential breeding material becomes a major concern.

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Aware of this urgent need, forest entomologists have tried persistently during the last three decades to find some indicator other than the empirically established, external characteristics of trees that would indicate susceptibility and to perfect methods to measure tree resistance reliably and directly. Besides other possible measurements of tree susceptibility the starch content in the phloem tissue, the pH reaction in the phloem sap or the conductivity of the outer sapwood for electrical current have been investigated. However, the most intensively studied indicators of the physiological condition of a tree were the osmotic pressure in the phloem (3, 6, 15) and the oleoresin flow (8, 9)[4]. These methods, however, have failed to produce specific criteria for tree susceptibility although they have revealed most interesting facts on the causes and mechanism of resistance in individual trees (15).

On the other hand, the empiric classification of susceptibility by external characteristics of trees used by foresters in selective cutting practices for centuries, has been developed into a risk-rating system for ponderosa and Jeffrey pine (7, 10), and applied with outstanding success (4)[5] to decrease losses from *Dendroctonus brevicomis* Lec. and *Dendroctonus jeffreyi* Hopk. The efficacy of this procedure, however, is restricted to the widely spaced old growth on the eastern slope of the Sierra Nevada Mountains where competition between trees ceases due to age and spacing and where the crown of a tree is likely to reflect the shape of its root system. With the progressive harvest of the virgin stands and the demand of forest management for short crop rotations, a system suitable for classifying risk in second growth stands becomes more desirable.

A possibility of providing a simple method for risk-rating pine seemed to be found in measurements of the oleoresin exudation pressure which represented in previous experiments both the state of water balance within a tree and the degree of resistance to infestation by subcortical-feeding insects (11, 12). Therefore, measurements of the o.e.p. were undertaken on a larger scale to determine whether the resistance of individual ponderosa pine trees could be expressed reliably as well as to test the applicability of such measurements as a technique for rating bark beetle risk in stands of second growth ponderosa pine. Also, since the extent of bark beetle infestations is known to depend on the population level (2, 6, 7), comparative investigations were conducted under epidemic as well as under endemic conditions.

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MATERIAL AND METHOD

The influence of o.e.p. on the resistance of ponderosa pine was investigated under epidemic conditions in the Crystal Bay area at the north end of Lake Tahoe in the State of Nevada. Here, in a triangular-shaped basin rising from the shore line at 6,000 feet elevation to a narrow pass at 8,000 feet, second growth ponderosa pine ranging from 55 to 75 years of age has been under attack by *Dendroctonus monticolae* Hopk. and *Dendroctonus brevicomis* Lec. since 1949.

This bark beetle infestation reached outbreak proportions in 1957 and continued at this high level through 1958–60, resulting in the loss of a considerable portion of the stand on nearly 4,000 acres. Usually the ponderosa pines were infested in groups of 3 to more than 100 trees, mostly in the vicinity of earlier infestations. Competition resulting from crowding in this overstocked stand was thought to be the major cause of the recent infestation. An estimated loss of more than 7,000 ponderosa pines in 1957, about 5,000 in 1958[6], 8,500 in 1959[7], and probably more than 12,000 in 1960 has resulted from this epidemic. Compared with the years prior to 1959, extremely dry conditions had persisted in 1959 and 1960. The season of 1960 was characterized by periods of hot weather in addition to the extreme drought. In these two years, the new infestation seemed to be concentrated in a belt running parallel to the shoreline through the center portion of the basin (Fig. 1). In this region, the site and stand were heterogeneous. Dry ridges stocked with clusters of small-sized timber changed to slopes and creek bottoms stocked with larger trees. Groups containing a large number of small trees were killed in the denser stands (Fig. 2), while, in general, groups containing only a few trees were killed on the better sites. *D. brevicomis* became more abundant in 1959 and 1960 than in the previous years, although *D. monticolae* still remained the predominant species in the summer infestation. Attacks by *D. monticolae* or mixed attacks of both species were more frequent than attacks by *D. brevicomis* alone. There were three to four times as many *D. monticolae* as *D. brevicomis* in these mixed attacks.

In both years of this investigation, measurements of the o.e.p. were taken from trees larger than 6″ d.b.h. These measurements were made simultaneously in several plots so as to coincide with the onset of the *D. monticolae* flight and the attack of the second generation of *D. brevicomis* in July.

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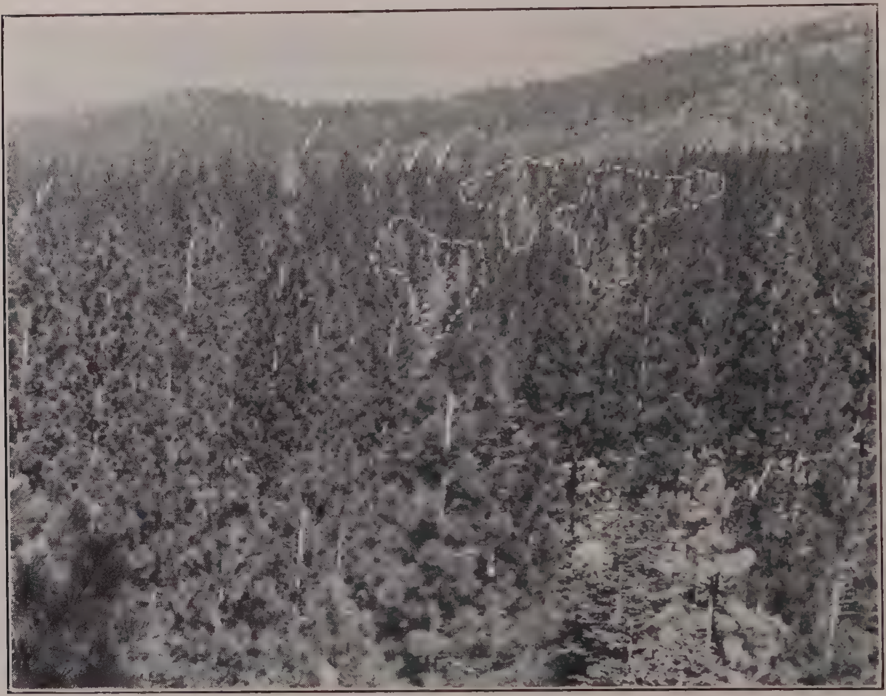

FIGURE 1. View of a portion of the bark beetle infested area at Crystal Bay, Nevada. Arrows and dotted lines indicate trees and groups of ponderosa pines recently killed by *Dendroctonus brevicomis* and *Dendroctonus monticolae*.

These plots were constituted as follows:

Plot A (1959)	Approximately 1.2 acres containing 121 ponderosa pines exhibiting good growth and widely spaced at the bottom of a gentle slope with a southeast exposure. Jeffrey pine comprised 5.0 per cent of the total stand.
Plot B (1959)	Approximately 2 acres on a ridge neighboring Plot A containing 259 ponderosa pines of fair to poor growth together with Jeffrey pine in the amount of 6.1 per cent of the total stand.
Plot C (1959)	A total of 44 ponderosa pines situated along the boundaries of Plots A and B when initial attack became apparent. These trees were included in the investigation for comparative purposes.
Plot B-1 (1960)	Approximately 1 acre containing 157 ponderosa pines of fair to poor growth within Plot B from 1959.
Plot D (1960)	A group of 201 selected ponderosa pines of fair to good growth on a southeast slope with Jeffrey pine comprising 45.8 per cent of the total stand.
Plot E	A group of 186 selected ponderosa pines of fair growth on a

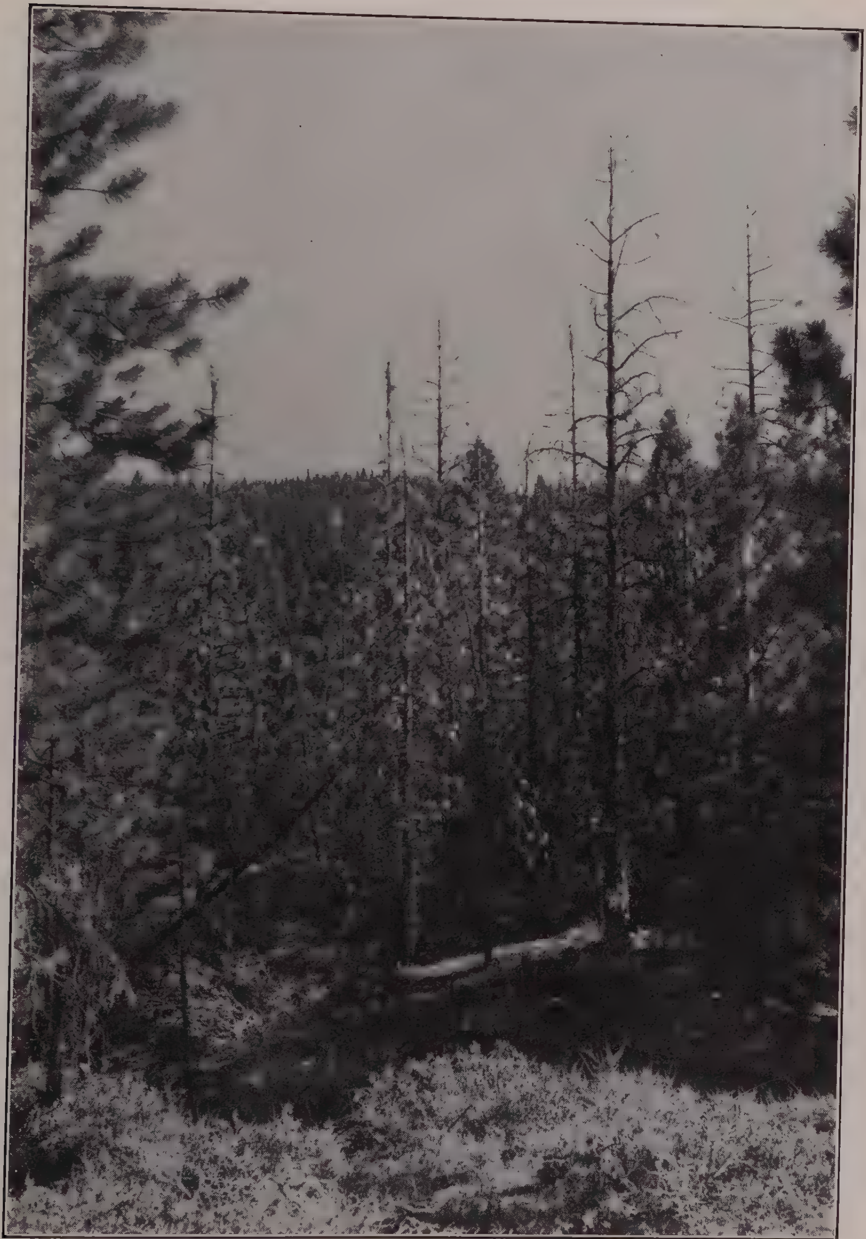

FIGURE 2. A group containing a large number of small sized trees killed by *Dendroctonus monticolae* and *Dendroctonus brevicomis* in 1958 near Plot B.

(1960) southwest exposed slope bottom. The surrounding stand is partially dense, but primarily open, spaced with Jeffrey pine in the amount of 76.4 per cent.

For comparative purposes, data were accumulated under endemic conditions. These o.e.p. measurements were taken in 80- to 100-year-old stands of second-growth ponderosa pine on the BTI Experimental Forest at Grass Valley, California, and the adjoining land of the New Verde Mines Co. These stands are situated in the foothills of the Sierra Nevada Mountains at an elevation between 2,100 and 2,500 feet. Each year these forests sustain moderate losses from infestations by *Ips confusus* (Lec.) and *Dendroctonus brevicomis*. In 1959, o.e.p. readings were taken on a total of 210 trees during July and August and on a total of 427 trees during the same months of 1960.

The basic method used in this investigation was described earlier (11). A hole 3/32 of an inch in diameter was drilled to a depth of 2 inches into the main bole, preferably at the north side of a tree and about 4 feet above the ground. A hydrostatic pressure gauge calibrated from 10 to 200 p.s.i. was inserted into the hole by means of a short nipple of $\frac{1}{8}$ inch pipe. The procedure was to attach one gauge to each tree in the late afternoon and to read the pressure the next morning at dawn when the o.e.p. was highest. On trees with low pressure, i.e. less than 20 p.s.i., this procedure was repeated with two gauges to compensate for mechanical errors. If more than one gauge was employed on a tree, the highest figure obtained was used for classification.

Upon measurement of the oleoresin exudation pressure, each tree was labeled with a metal number. At the end of the summer it was determined whether or not the tree was attacked and whether or not attacked trees succumbed or survived. The attack intensity was also measured. The determination of the intensity of attack consisted in counting the number of visible entrances in a three-foot wide strip around the lower bole wherever the attack was heaviest. Measuring the circumference at the center line of the strip observed provided the basis for the reduction of the total counted attacks to the number per square foot of bark surface. The range of 0.1 to 0.3 entrances per square foot is referred to hereafter as single attack, 0.4 to 2.9 as scattered attack, and above 3 attacks per square foot as mass attack.

RESULTS

As shown in Table I, there was a close correlation between bark beetle infestation and o.e.p. of the host tree even during a season when the extent of bark beetle infestation was extremely severe.

Under epidemic conditions. The incidence of attack on comparably situated trees with low (<4 atmospheres) and high (>4 atmospheres) pressure was very similar, i.e. 32 per cent and 32.7 per cent in 1959 and 39.4 per cent and 37.8 per cent in 1960 respectively. In both years, the majority of trees with o.e.p. higher than 4 atmospheres survived. In 1959, from the attacked ponderosa pines with high o.e.p., only 2.9 per cent were killed, while in 1960 16.3 per cent were killed. The opposite situation is reflected in the trees with o.e.p. less than 4 atmospheres. Here, from 60 trees attacked in 1959 14.3 per cent survived while only 8.6 per cent survived in 1960.

In this comparison, the choice of separation at 4 atmospheres is arbitrary. However, there exist two reasons for this choice. In previous investigations (11, 12), 4 atmospheres were found to be the minimum pressure in ponderosa pines in a normal physiological condition. In addition, it was found that the instruments more accurately recorded pressures above 4 atmospheres than below this point.

TABLE I

Incidence of Attack by Dendroctonus brevicomis and Dendroctonus monticolae on Ponderosa Pines with Different Oleoresin Exudation Pressure in the Crystal Bay Area of Nevada during Two Seasons

Oleoresin exudation pressure of trees	1959 Observations				1960 Observations			
	No. of trees	Percentage of trees			No. of trees	Percentage of trees		
		Unattacked	Attacked and surviving	Killed		Unattacked	Attacked and surviving	Killed
Less than 4 atm.	186	68.0	14.3	17.7	336	60.6	8.6	30.8
More than 4 atm.	238	67.3	29.8	2.9	208	62.2	21.5	16.3
Total	424	67.5	23.1	9.4	544	61.0	13.5	25.5

The climatic differences between 1959 and 1960 were expressed in both the population level of the two bark beetle species, and in the physiological condition of the ponderosa pines. The o.e.p. was generally higher in the trees measured in 1959 than in 1960. In 1959, 57 per cent of the trees had more than 4 atmospheres o.e.p. as compared to 38 per cent in 1960. From 111 trees measured in 1959 and remeasured in 1960, the number of trees with 0 atmospheres was 30 and 35 respectively, <4 atmospheres 30 and 34, and >4 atmospheres 51 and 42. The much higher population level of beetles in 1960 was indicated by the larger number of trees attacked. Also 28 per cent of all trees were mass attacked compared with only 14 per cent in 1959.

Furthermore, in each of the years, local differences in susceptibility due to stand and site conditions became apparent (Table II). In 1959, a stand of widely spaced ponderosa pine of good growth at a slope bottom (Plot A) showed a similar rate of attack (24.8 per cent) as a neighboring stand (Plot B) of fair to poor growth on a dry ridge (24.7 per cent) but only half as much loss from bark beetles was recorded (3.3 per cent in A and 6.6 per cent in B). Also, Plot B contained twice as many trees with an o.e.p. of less than 4 atmospheres as Plot A. A portion of Plot B was compared in 1960 (Plot B-1) with one plot exhibiting good to fair growth characteristics (Plot D) and another exhibiting only fair growth characteristics (Plot E). In Plot B-1, 46.3 per cent of the trees were attacked and 43.2 per cent in Plot E, but only 29.5 per cent in Plot D. The mortality of trees due to successful bark beetle invasion was similar in Plots B-1 and E, with 32.3 per cent and 26.1 per cent respectively, but lower in Plot D with 15.1 per cent. Although Plot D contained a relatively dense stand, the better site conditions were again expressed in the total number of trees with an o.e.p. of 4 atmospheres and greater. This amounted to 49 per cent in Plot D, but only 36 per cent in Plot B-1, and 28 per cent in Plot E.

TABLE II

SEVERITY OF ATTACK BY DENDROCTONUS BREVICOMIS AND DENDROCTONUS MONTICOLAE ON PONDEROSA PINE GROWING ON FOUR SITES UNDER CONDITIONS THAT WERE CONDUCIVE TO DIFFERENCES IN OLEORESIN EXUDATION PRESSURE

Conditions on site					Total No. of trees	No. of trees with o.e.p.	Percentage of trees		
Plot No.	Year of experiment	Type of site	Spacing	Growth rate			Unattacked	Attacked and surviving	Attacked and killed
A	1959	Bottom slope, SE exposure	Open	Good	*121*		*75.2*	*21.5*	*3.3*
						29 <4 atmos.	79.3	13.8	6.9
						92 >4 atmos.	73.8	23.9	2.3
B	1959	Ridge	Dense	Fair/poor	*259*		*75.3*	*18.1*	*6.6*
						131 <4 atmos.	78.7	10.0	11.3
						128 >4 atmos.	71.7	26.6	1.7
B-1*	1960	Ridge	Dense	Fair/poor	*157*		*53.7*	*14.0*	*32.3*
						101 <4 atmos.	52.5	7.9	39.6
						56 >4 atmos.	55.3	19.7	25.0
D	1960	SE slope	Open/dense	Good/fair	*201*		*70.5*	*14.4*	*15.1*
						102 <4 atmos.	72.6	7.8	19.6
						99 >4 atmos.	68.8	21.1	10.1
E	1960	Bottom slope, SW exposure	Open/dense	Fair	*186*		*56.8*	*17.1*	*26.1*
						133 <4 atmos.	57.1	9.8	33.1
						53 >4 atmos.	56.6	24.5	18.9

* Only a portion of plot B was remeasured and observed in 1960.

Under endemic conditions. The population level of bark beetles in the area investigated did not permit the comparison of actual beetle attack, as only two of the trees measured had been attacked successfully. However, this experiment gave some valuable figures as to the distribution of o.e.p. in undisturbed second growth stands in two subsequent years, as well as in a previously heavily logged area (Table III). In 1959 in the undisturbed stands (A), 91 per cent of the pines exhibited high o.e.p., and only 9 per cent an o.e.p. less than 4 atmospheres. A pine stand (C) of the same age and on a comparable site, where more than one-half of the original stand had been removed the previous spring, consisted of 18 per cent low and 82 per cent high pressure trees.

In 1960, continuous drought led to a considerable decrease of o.e.p. in the same second growth ponderosa pine stand (B) that was investigated in 1959. Only three-fifths of the trees investigated exhibited a high o.e.p. but with an average of only 7.2 atmospheres (1959: 9.1 atmospheres). However, among the 427 ponderosa pines observed, there existed only one group of 9 trees with zero o.e.p., 4 of which had been scarred by lightning during the previous spring. Two of the trees which had been struck by lightning were successfully attacked by *D. brevicomis.*

TABLE III

RELATIVE CLASSIFICATION OF PONDEROSA PINE, AGES 80 TO 100 YEARS, ACCORDING TO OLEORESIN EXUDATION PRESSURE ON TWO SITES AND UNDER DIFFERENT STAND CONDITIONS AT GRASS VALLEY, CALIFORNIA, DURING TWO DIFFERENT SEASONS

Stand and location	Date	Total No. of trees	No. of trees with oleoresin exudation pressure in atmospheres of												
			0	1	2	3	4	5	6	7	8	9	10	11	12
A. Undisturbed, North Star Exp. Forest	July/ August 1959	80	2	1	1	3	5	2	4	4	3	15	21	15	4
			(=9%, average 1.7 atm.)				(=91%, average 9.1 atm.)								
B. Undisturbed, North Star Exp. Forest	July/ August 1960	427	82	39	27	22	22	25	36	62	53	32	23	3	1
			(=40%, average 0.9 atm.)				(=60%, average 7.2 atm.)								
C. Heavily logged, New Verde Mines Co.	August 1959	130	4	5	5	10	8	12	8	5	16	22	19	14	2
			(=18%, average 1.9 atm.)				(=82%, average 8.2 atm.)								

Initial attack and mass attraction. The data further suggest that there existed an interrelationship between successful initial attack and an increased attraction as evidenced by mass attack which became most obvious in the Crystal Bay study. Under the lower population level in 1959, in the low o.e.p. range, more than half (34 trees) of the 60 initially attacked trees became mass attacked. Less than one-third, i.e. 24 of the 78 trees initially attacked became mass attacked when they had an o.e.p. greater than 4 atmospheres. In both groups nearly one-third of the trees observed were initially attacked. Under the higher population level in 1960, 78 per cent of the initially invaded trees became mass attacked in the low o.e.p. range but only 43 per cent of the trees with an o.e.p. of 4 atmospheres or greater were mass attacked. Again, the frequency of initial attack was very similar, 39 per cent in low pressure trees and 38 per cent in trees with an o.e.p. of 4 or more atmospheres.

The data obtained in 1959 further substantiate the occurrence of the mass attraction (Table IV). The trees observed were classified into three groups; low (less than 4 atmospheres), medium (4 to 6.5 atmospheres) and high o.e.p. (6.6 to 12 atmospheres). All groups received initial attack in a similar magnitude, i.e. 31 to 36 per cent. However, only 13 per cent or 6 of the 45 initially invaded trees in the high o.e.p. range were mass attacked, compared with 57 per cent in the low and 54 per cent in the medium o.e.p. ranges. Similarly, the plots with the largest number of trees with low o.e.p. contained the largest number of mass attacked trees (Table II). It appears that the more breeding material that is available and receives successful initial attack, the more attractive this material becomes to the bark beetle population in that area (1, 14).

TABLE IV

TOTAL NUMBER AND PER CENT OF TREES ATTACKED AND UNATTACKED BY DENDROCTONUS BREVICOMIS AND DENDROCTONUS MONTICOLAE IN EXPERIMENTAL PLOTS IN THE CRYSTAL BAY AREA DURING 1959 IN RELATION TO THE RANGE OF O.E.P. AND THE INTENSITY OF BEETLE ATTACK

Range of o.e.p. in atmospheres	Total No. of trees	No. of trees unattacked	Number of trees			
			With scattered attack*	With mass attack**	Killed by mass attack	Percentage of trees attacked
Less than 4 (low o.e.p.)	186	126	26	34	33	32
4 to 6.5 (medium o.e.p.)	91	58	15	18	6	36
6.6 to 12 (high o.e.p.)	147	102	39	6	1	31
Total No. of trees	424	286	80	58	40	

* Less than 3 entrances per square foot bark surface.
** Three and more entrances per square foot bark surface.

Relative resistance of infested trees. Further observations confirmed the relative resistance of ponderosa pines after successful mass invasion had occurred. At the end of July, the o.e.p. of 26 mass attacked trees ranged between 1.5 and 6.5 atmospheres; 14 of these trees proved resistant and exhibited, when remeasured on September 1, similar pressures as before with but one exception. The remaining 12 trees proved susceptible and the o.e.p. ceased in 10 of these within the next few weeks. However, two trees retained some pressure. There was a definite difference in the development of the *D. brevicomis* brood. Both trees contained only a few larvae and eggs; some of the larvae had perished in the oleoresin flow, while most of the other 10 trees mass attacked at the same time already contained callow adults, pupae, and some late instar larvae. There was also an indication that the oleoresin flow, in addition to delaying egg deposition and development, strongly influenced the rate of oviposition. Trees with o.e.p. less than 1 atmosphere contained more larvae per unit of bark surface than trees with 1.5 to 6.5 atmospheres. Samples taken from 4 trees with 1.5 to 6.5 atmospheres averaged 22.5 individuals per square foot while trees with the o.e.p. less than 1 atmosphere had 54 individuals per square foot. This observation confirms the concept that "the number of offspring produced in vigorous growing hosts" is usually smaller than in more suitable breeding material (1, p. 210).

DISCUSSION

It is commonly thought that under epidemic conditions the more aggressive bark beetle species are able to overcome the host tree regardless of its physiological state (7), while under endemic conditions susceptible trees become the only suitable breeding material. The study presented confirms this assumption. However, when compared with other coniferous species, it shows that individual ponderosa pines in a normal physiological state exhibit a high degree of resistance to the invasion of aggressive bark beetles even under outbreak conditions.

The investigations further suggest the strong dependency of tree susceptibility on site, stand, and weather conditions. An example is the study in Crystal Bay area. Although the infestation was distributed throughout the entire forest, the outbreak of *D. brevicomis* and *D. monticolae* occurred with increasing severity in various plots and different years due to the physiological state of the trees. From the data obtained in stands under epidemic and endemic conditions it can be assumed that at least 20 per cent of the trees in second growth ponderosa pine have to be in a subnormal condition before *D. brevicomis* and *D. monticolae* are permitted to build up to outbreak proportions.

In other words, the previous experiments (11) and this study confirm the concept that bark beetle outbreaks occur only where, and as long as, suitable breeding material is available. Less suitable material may be attacked successfully under high population pressures but it proves less favorable for oviposition and brood survival. However, the disturbances created in a stand during an outbreak should not be overlooked. The sudden exposure of trees near those killed by bark beetles decreases their turgidity, at least temporarily, due to higher transpiration which in turn reduces the o.e.p. and innate resistance (11, 12).

As a preventive measure, the removal of material susceptible to bark beetle infestation has been recommended during the past decades by many forest entomologists. Following this recommendation, the risk-rating systems developed by Salman and Bongberg (10) and Keen and Salman (5) have been employed with remarkable results in virgin growth ponderosa and Jeffrey pine in the eastern range of the Sierra Nevada Mountains (4). This method is no more than 70 to 85 per cent effective over a 10-year period in eliminating high risk trees under endemic conditions (13, p. 10).

Furthermore, it has not been effective on the westside Sierra type and in second growth stands. Therefore, a method such as measuring o.e.p. which represents a similar effectiveness under outbreak conditions could be put to use with comparable advantage not only in second growth but on the western slope of the Sierra Nevada Mountains.

The technique of measuring o.e.p. is very simple, especially when compared with other evaluations of tree vigor. For extensive use, however, the method of measuring pressure would have to be perfected.

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STUDIES ON THE HOST SELECTION BEHAVIOR OF IPS CONFUSUS (LeCONTE) (COLEOPTERA: SCOLYTIDAE) ATTACKING PINUS PONDEROSA[1]

David L. Wood[2] and J. P. Vité[3]

SUMMARY

An investigation of the host selection behavior of *Ips confusus* (LeConte) attacking cut host material of *Pinus ponderosa* Douglas was performed at the Boyce Thompson Institute Forest Research Laboratory, Grass Valley, California. Observations of the attack pattern in nature revealed an initial period which was characterized by a few successful beetle penetrations occurring at various intervals after cutting and always preceding (usually 1 to 6 days) an abrupt increase in the attack intensity. This sudden increase in the number of attacks per unit of bark surface following the initial or pioneer attack has been termed the mass attack. To test the hypothesis that in susceptible host material the initially attacking beetles create a secondary attraction to which a large proportion of the local population responds, manipulation of the pioneer attack was attempted. A technique was developed whereby newly attacked samples of bark containing the initial gallery and the beetle were excised and affixed to other felled trees in various experiments. The first strikes from the local population were recorded on the tree possessing these excised galleries before 24 hours had elapsed in all experiments. Subsequent tests in which the mass invasion was successfully advanced and delayed proved that this phenomenon was not a chance event but the result of an attraction created by successful initial attack.

A series of experiments was performed in an attempt to isolate the source of this secondary attraction. Exposure of the sapwood and phloem independently did not influence attractiveness. Where bark samples containing forced male and female attacks were excised and fixed to separate trees, a positive response by the attacking population was recorded on the tree with bark samples containing male beetles. Initial galleries from which the adults had been removed and forced attacks containing teneral adult males did not create an attraction. The theory proposed by Person that the secondary attraction (mass attack) is the result of a fermentation produced by yeasts introduced into the inner bark by the pioneer attacking beetles is discussed in the light of the results obtained in this study.

Secondary attraction created by initial attack is probably the result of a complex of factors, but nevertheless is intimately associated with the presence of the live, mature male within the initial gallery. Both males and females of the attacking population exhibit a positive response to the presence of the male.

Variation in the number of attacks at the onset of the mass attack and in the maximum attack intensity recorded in a 24-hour period, probably reflects the relative population level at the advent and locale of the test.

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INTRODUCTION

Insect host selection behavior is probably one of the most important and little understood facets of the host-insect relationship. With regard to bark beetles many investigators (1, 6, 7, 8, 9, 11, 12; and footnotes[4–9]) have contributed significant findings in this research area. However, there is still a considerable lack of knowledge concerning the mechanisms or processes underlying host selection behavior.

The observations and experiments reported in this paper are part of a study designed to determine and assess the factors or processes that attract bark beetles to susceptible trees. This work was performed at the Boyce Thompson Institute Forest Research Laboratory, Grass Valley, California, during the spring and summer months of 1958–1960. Initial studies were conducted with the California 5-spined ips, *Ips confusus* (LeConte). The reason for this choice was based primarily on the life habits of this insect, i.e., it attacks both standing as well as cut trees. Also, high populations are usually present in the experimental forest for about 6 months of each year and 4 to 5 generations are produced each season. All trees and cut material used in this study were *Pinus ponderosa* Douglas.

PATTERN OF ATTACK

Felled tree observations. Mass attack has been observed to occur with such bark beetle species as *Dendroctonus brevicomis* Lec. (7), *D. monticolae* Hopk. (11), and *Ips pini* (Say) (1). Repeated observations over the past three years have shown conclusively that *I. confusus* exhibits the same behavior as that reported for other bark beetle species attacking members of the genus *Pinus*. However, in order to determine the entire pattern of attack as it occurs in nature, a ponderosa pine was felled and a record of the number of attacks occurring each day was maintained (Fig. 1).

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Straight pines were used to mark the beetle's entrance tunnel. To obtain maximum accuracy, counts were performed in the early morning. The accumulated boring dust from attacks that had occurred during the previous day was more evident at this time because attacking beetles had been working in the inner bark for 12 hours and longer.

The first attacks were observed on the second day after cutting. During the sixth day there was a 4.5-fold increase in the number of strikes recorded during the previous day. The number of new attacks increased to the highest level on the seventh day. Because counts were not recorded on the ninth and tenth days, the number of attacks indicated on the eleventh day reflects the cumulative number for that three-day period. In all probability 75 per cent of these attacks occurred on the ninth day. Further reference to the mass attack in this paper denotes this large influx of beetles similar to that which is indicated in Figure 1. This should not be confused with the mass attack described by Struble and Hall (10) in which reference is made to the mass feeding habit of *I. confusus*. In this instance adults feeding en masse concentrate in a relatively small area of the inner bark. Broods are rarely produced. The initial or pioneer attack refers to the first few attacking beetles observed penetrating the bark prior to the abrupt increase.

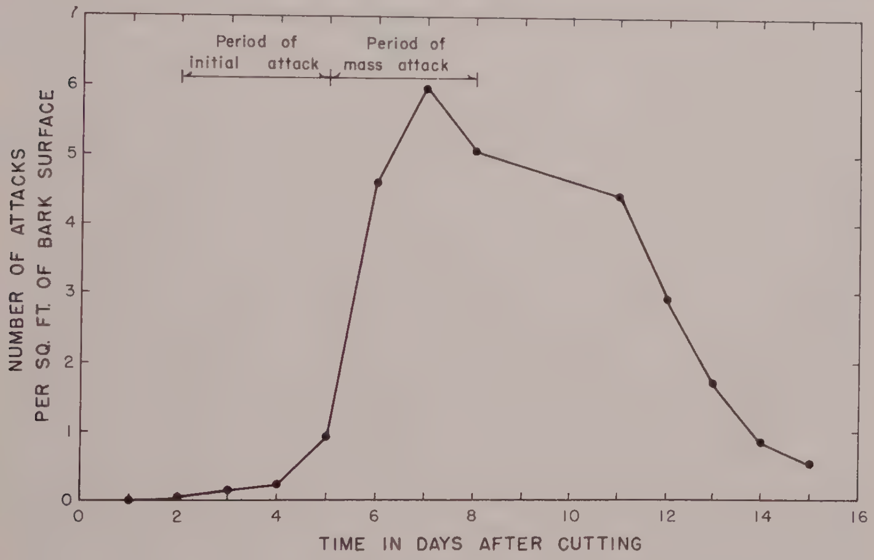

Figure 1. Frequency of attacks by *Ips confusus* on a felled ponderosa pine, dbh 12 in., length 35 ft., showing the initial attack followed by mass attack.

The most consistent element of the pattern shown in Figure 1 was the sudden increase in the number of attacks as evidenced on the fifth day.

This statement is supported further from numerous other observations on cut host material. The appearance of the initial attacks has been observed to vary from the first day after cutting to a period of 4 to 6 weeks. The time between cutting and initial attack appears to be related to the population level, the amount of host material available to that population (12), and weather conditions. The occurrence of the mass attack varied from 1 to 6 days after the initial attack had commenced. In rare instances no mass attack was evident, perhaps due to extremely low population levels at the time of the test, or to competition with more attractive host material in the vicinity of the test. Mass attacks were invariably preceded by initial attacks by a few individuals. The magnitude of the mass attack varied and was probably dependent upon the insect populations present at a given time in a given area.

From the standpoint of attraction, the most significant aspect of the attack pattern was that an initial attack by a few beetles always preceded an abrupt increase in the number of strikes per unit of bark surface. The following working hypothesis was derived from the above observations: in susceptible host material, the initially attacking beetles create a strong attraction to which a large portion of the local population responds in a relatively short period of time. Person (7) presented essentially the same idea and suggested that the secondary attaction was created by yeasts introduced into the inner bark by the pioneer beetles. From Figure 1, the 350 per cent increase in the number of attacks on the fifth day indicates the period of greatest attraction. The results of numerous tests have shown that the mass attack pattern was similar to that shown in Figure 1. These data indicate that the period of greatest attack intensity was generally of very short duration (24 hours) and did not increase significantly after the first day's large influx of beetles.

Latin square experiments. Further evidence is presented to support the original observation that an attraction is created by successful initial attacks of *I. confusus*. The results of two experiments designed to evaluate the relative attractiveness of logs cut from various portions of the tree are presented in Table I. Three-foot sections were removed from the crown and trunk of the same tree. Each test consisted of 3×3 Latin squares containing trunk sections, crown sections with limbs, and crown sections with the limbs removed. The analysis of variance gave no significant evidence of differences among the attacks associated with the treatments. The data could support the idea of random selection as evidenced by the beetles initiating attacks in Test 1 on August 27 and in Test 2 on August 28. However, the attacks which occurred subsequent to these pioneer attacks were the result of a directed response to the preattacked samples. In Test 1 all five of the initially attacked logs received further attacks while the remaining four samples were unattacked throughout the length of the test.

This occurred despite the relatively short distance (25 ft.) between samples. The same pattern was exhibited in Test 2, but two samples which were initially attacked on the second day of the test did not receive any further attack.

Caged log experiments. The following experiments were performed to evaluate the role of the initial attacks and resulted in a similar attack pattern. Four log samples cut from a ponderosa pine were placed adjacent to a cage containing three samples from the same tree. All logs were cut in three-foot lengths. Two of the four samples received initial attacks on the second day following cutting and the remaining two on the third day (Table II, Test 3). Four days after the test was initiated, the unattacked caged logs were exposed to the attacking population. The preattacked logs continued to receive attacks for eight days while the newly exposed unattacked samples remained unattacked during the same period.

TABLE I

DISTRIBUTION AND NUMBER OF ATTACKS ON SAMPLES OF PONDEROSA PINE FROM THE TRUNK (B), THE CROWN (L), AND THE CROWN WITH THE LIMBS REMOVED (W) IN TWO LATIN SQUARE TESTS WITH DISTANCES OF 25 AND 50 FEET BETWEEN SAMPLES

Date and row	Test 1 (25 feet)			Test 2 (50 feet)		
Row 1	B	L	W	L	W	B
Aug. 27	0	2	4	0	0	0
28	0	1	0	1	0	1
29	0	0	3	0	0	0
Sept. 9	0	5	14	0	0	0
Row 2	L	W	B	B	L	W
Aug. 27	0	0	1	0	0	0
28	0	0	0	2	0	0
29	0	0	1	0	0	0
Sept. 9	0	0	16	19	0	0
Row 3	W	B	L	W	B	L
Aug. 27	1	5	0	0	0	0
28	1	3	0	0	0	0
29	0	3	0	0	0	0
Sept. 9	4	12	0	0	0	0

Another test initiated later in the summer revealed the identical pattern (Table II, Test 4). The technique was the same as that which was employed in the above experiment except that larger (5 ft.) log samples were used. The logs which were not preattacked remained unattacked subsequent to the removal of the cage (42 days), while of the two preattacked samples one was continuously attacked throughout this period except for a 10-day interval in September.

TABLE II

DAILY RECORD OF ATTACKS BY IPS CONFUSUS ON PREATTACKED AND UNATTACKED LOG SAMPLES OF PINUS PONDEROSA IN EARLY (TEST 3) AND LATE (TEST 4) SUMMER

Days after initiation		No. of attacks on						
		Preattacked logs not caged				Unattacked logs caged first four days (Test 3) and first nine days (Test 4)		
Test 3	1	0	0	0	0	—	—	—
	2	0	0	3	4	—	—	—
	3	3	1	3	3	—	—	—
	4	5	2	13	3	Cage removed		
	5	5	3	15	2	0	0	0
	6	0	2	1	0	0	0	0
	7	1	0	7	2	0	0	0
	8	4	2	2	4	0	0	0
	9	0	1	1	1	0	0	0
	10	2	1	0	0	0	0	0
	11	4	0	0	1	0	0	0
	12	6	2	0	1	0	0	0
Test 4	1	0	0			—	—	
	3	1	0			—	—	
	7	0	2			—	—	
	9	0	9			Cage removed		
	10	0	3			0	0	
	11	0	3			0	0	
	12	0	0			0	0	
	14	0	5			0	0	
	16	0	2			0	0	
	18	0	3			0	0	
	27	0	1			0	0	
	30–40	0	0			0	0	
	41	0	3			0	0	
	46	0	9			0	0	
	47	0	1			0	0	
	48	0	0			0	0	
	51	0	4			0	0	

Each of these experiments supports the observation that host material becomes more highly attractive to the local population only after it receives an initial attack by a few beetles. This attraction was so dominant that not a single attack was recorded on the host material not preattacked, although it was cut from the same tree at the same time and located not more than two to three feet from the preattacked samples. The close proximity of these samples to each other excludes the possibility that the preattacked and caged samples may not have been exposed to the same attacking population throughout the course of each experiment.

MANIPULATION OF THE INITIAL ATTACK

To test further the hypothesis that the pioneer attacks induce the mass attack, experiments were designed in an attempt to both advance and delay the occurrence of the mass attack. If this mass influx of beetles is a chance event, then manipulation of the initial attack should not influence the periodicity of the mass attack.

Method. Paired felled tree tests were used in most experiments. To facilitate counting, the trees were elevated three feet from the ground on steel posts. In each test two trees of similar size and crown ratio were cut and placed in a parallel position under as similar exposure conditions as practicable (Fig. 2). However, the intervals between trees varied. Initially attacked areas of bark were excised with a good chisel or an archpunch. These bark sections were either two inches square or two inches in diameter and were cut deep enough so as to include a half inch section of the sapwood. Each bark section, therefore, contained a single beetle gallery which had been initiated within the 24-hour period prior to the beginning of the experiment. The bark sections were removed so as to maintain the beetle's activity within the initial gallery. Further reference to "excised, initially attacked bark samples" will denote a sample of bark and sapwood containing a newly established gallery together with the beetle removed completely intact. Straight pins were used to mark the beetle's entrance tunnel during each count.

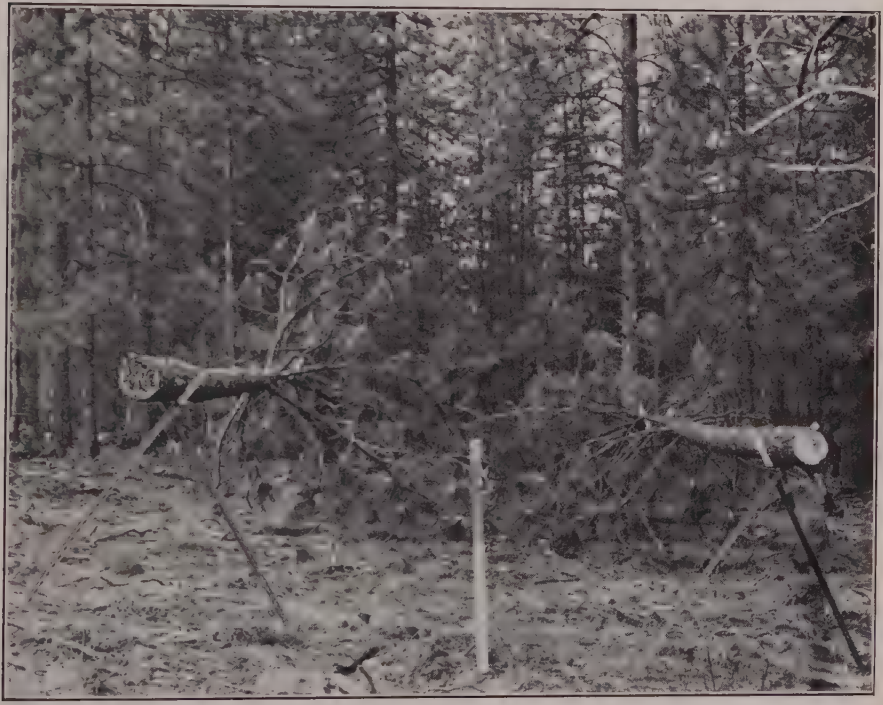

FIGURE 2. Method of arrangement of paired tree experiments. Trees are elevated above the ground on steel posts to facilitate examination on all sides.

Advance of the mass attack. Thirty initially attacked bark samples were removed from another source and affixed to the bole of a tree felled on the same day. The control tree was located parallel to the experimental tree at a distance of 21 ft. in Test 5, 45 ft. in Test 6, and 150 ft. in Test 7. The resultant attack patterns are shown in Table III. In all experiments the excised bark samples containing an initial gallery attracted further attacks on the first day after initiation of the test, while the first attacks recorded on the controls were delayed 1, 3, and 7 days in Tests 5, 6, and 7, respectively. There also appeared to be a definite correlation between the length of the delay and the distance between treatment and control. However, it should not be overlooked that the population level at the time of each test may also have had some influence on the results obtained.

TABLE III

ADVANCE OF MASS ATTACK—ATTRACTION CREATED BY EXCISED BARK SAMPLES, INITIALLY ATTACKED, AND THE INFLUENCE OF DISTANCE BETWEEN TREES WITH AND WITHOUT THESE BARK SAMPLES ON THE DISTRIBUTION AND TIME OF SUBSEQUENT ATTACKS

Days after initiation	Number of attacks per sq. ft. of bark surface, in tests with various distances between trees, with					
	Test 5 (25 feet)		Test 6 (45 feet)		Test 7 (150 feet)	
	Excised initially attacked bark samples	Control	Excised initially attacked bark samples	Control	Excised initially attacked bark samples	Control
1	0.54	0	0.10	0	0.08	0
2	4.94	0.01	0.28	0	0.67	0
3	6.69	8.72	1.06	0	0.42	0
4	3.77	7.01	1.91	0.27	0.42	0
5	3.62	6.04	2.02	0.74	0.27	0
6	—	—	1.08	0.93	0.63	0
7	—	—	0.30	0.27	0.08	0
8	—	—	0.10	0.08	0.03	0.03

These tests were performed not only in an attempt to influence the mass attack but also to determine what effect the distance between control and treatment had on the attack pattern. It was essential in this study to expose both trees to the same local population at the same time in order to be assured that any differences recorded were a result of the imposed experimental conditions. If these criteria were not satisfied, then apparent differences recorded at large distances would be invalid. Conversely, if the trees were too close together, differences in attraction would not be easily discernible. In one test in which an interval of less than three feet was used, no differences were recorded, but in later experiments where 21 ft. and 45 ft. intervals were used, consistent differences were observed.

Similarly, attacks have been recorded on the third day after cutting while host material cut on the same day but one-half mile distant remained unattacked for a period as long as eight weeks.

The mass attack in Test 5 began on the second day and reached its peak on the third day, while on the control the highest point occurred also on the third day, but with only a one-day preattack period. This strongly indicates that the mass attack on the tree possessing the excised initial galleries was partially dissipated on the control and was probably a result of the close proximity (21 ft.) of the control to a highly attractive location.

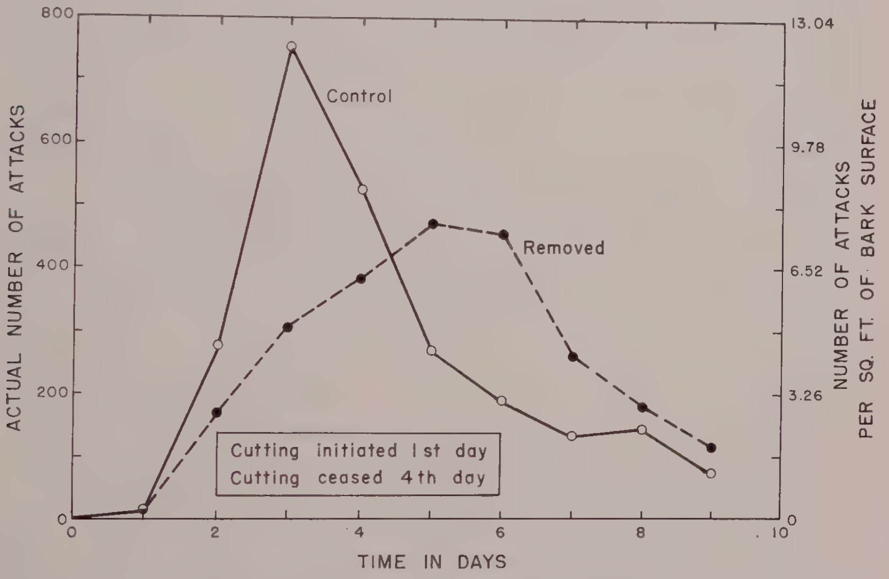

Figure 3. Delay of the mass attack. Results of a paired tree experiment where the newly initiated galleries were excised from one tree for the first three days of the test. The attack pattern indicates both a delay and prolongation of the mass attack.

The same effect was exhibited in Test 6 but to a lesser degree. Here there was some evidence of a prior build-up on the control. Also, the highest attack intensity (0.93 attack per sq. ft.) occurred one day later than on the tree possessing initial attacks. Little or no influence was exerted on the control by the more attractive host material to which excised initial galleries had been attached in Test 7. A peak was recorded on both the second and sixth days, while the mass attack was not evidenced on the control until 17 days had elapsed. The control received attacks for nine days prior to the mass attack.

The mass attack was advanced in time over the control in all tests, irrespective of the spacing employed. Paired tree tests in which excised initial galleries were not attached to either tree were initiated at various intervals during the same period that the above experiments were in progress. No attacks were recorded in the first three days after cutting and in many cases a week or more had elapsed. On two occasions more than five weeks passed before the first attacks were recorded. The first attacks from natural populations on the test trees all occurred within 24 hours. These experiments showed conclusively that an attraction was associated in some manner with the excised, one-day-old beetle galleries.

Delay of the mass attack. Twenty excised, initially attacked bark samples were attached to each of two felled trees as previously described. A ten-foot spacing between trees was utilized. This procedure was followed in order to attract simultaneous attacks from the local population on both samples. At 8:00 a.m. on the first day after initiation of the experiment, 13 and 14 attacks were recorded on the two trees. At this time the excised, attacked bark sections were removed from both samples. All newly initiated galleries were then excised from one tree at one- to two-hour intervals and counts were performed on the control at the same time. The results of removing the beetle galleries as they were initiated during the first three days of the test are shown in Figure 3.

TABLE IV

DELAY OF MASS ATTACK INDUCED BY THE PERIODIC REMOVAL OF NEWLY INITIATED GALLERIES FOR THE FIRST THREE DAYS FROM ONE OF TWO TREES

Days after initiation	Cumulative percentage of total number of attacks on	
	Tree from which the beetles and their galleries were removed	Control
2	8	12
3	21	44
4	37	66
5	57	77
6	76	85
7	88	91
8	95	97

The peak of the mass attack occurred on the control during the third day of the experiment, reaching an intensity of 12.24 attacks per sq. ft. The greatest intensity of attack on the treated tree (7.66 attacks per sq. ft.) was delayed until the fifth day. Therefore, the peak of the mass attack was delayed two full days by excising the galleries soon after they were initiated. Further evidence of this delay is shown in Table IV. On the fourth day, 66 per cent of the total attacks on the control had occurred while at the same time only 37 per cent of the total had been recorded on the treated tree. By the seventh day, the invasion reached 91 and 88 per cent of the accumulative total on the control and treated tree, respectively. The total number of attacks recorded on each tree was nearly identical (treated tree = 2354, control = 2377). Since both trees in this experiment contained the same bark surface area, comparisons of actual numbers of attacks were considered valid.

The mass attack was interpreted as commencing on the day that the greatest increase in attack intensity was recorded. The greatest 24-hour increase on the control was 478 attacks compared to an increase of 157 attacks on the treated tree, occurring on the third and second days, respectively. Therefore, the period of the mass attack was lengthened to four or five days in comparison to two days on the control. The pattern of attack was so altered on the treated tree that not only was the peak of the mass attack delayed, but its duration was also increased, compensating for the lower attack intensity recorded during the mass attack period. A preliminary experiment designed as described above revealed the same pattern.

LOCALIZATION OF SOURCE OF ATTRACTION

Because all evidence accumulated to this point supports the original observation that a mass attraction is created by or associated with the initially attacking beetles, a series of experiments was designed in an attempt to isolate the source of this attraction. Paired tree experiments were employed as already described.

Exposure of sapwood. In previous experiments, bark sections containing a single recent attack were excised from another source and affixed to the bole of a newly felled tree. The exposed sapwood which consequently accompanied each of these initial galleries possessed an unknown role in the differential attraction which was created in these experiments. To evaluate this unknown factor, an experiment was initiated which was comprised of three trees felled at 21-foot intervals. Twenty excised, initially attacked bark samples were affixed to the center tree in the same manner as employed previously. At the same time, 20 unattacked bark samples were removed from the same source and attached to another tree. No bark samples were attached to the third tree. The resultant attack pattern (Table V) was similar in most respects to that which was recorded in previous tests (Table III). The tree with bark samples containing initial galleries received the greatest number of subsequent attacks during the first three days and was mass-attacked on the fourth and fifth days, while the control was very lightly attacked during the first three days and did not receive a mass attack until the fifth day of the experiment. These data parallel very closely the pattern expressed in Tests 5, 6, and 7, proving again that these initial galleries are creating some attraction to which the local population is responding

The tree on which excised bark samples without beetles were attached

TABLE V

COMPARISON OF THE RELATIVE ATTRACTION CREATED BY INITIALLY ATTACKED AND UNATTACKED BARK SAMPLES EXCISED FROM THE SAME TREE TO DETERMINE THE ROLE OF EXPOSED SAPWOOD IN CREATING AN ATTRACTION

Days after initiation	Number of attacks per sq. ft. on felled trees with		
	Excised initially attacked bark samples	Excised unattacked bark samples	Control
0	0.11	0	0.02
1	0.30	0	0.02
2	1.67	0	0.47
3	10.64	0	6.09
4	10.64	1.43	18.26
5	—	21.66	—

did not receive any attacks until the fifth day and was not mass-attacked until the sixth day. This result would support the conclusion that no attraction was associated with the exposed sapwood. Had there been some attraction created, the pattern would have more closely resembled that which was recorded on the control. Moreover, both the control and the tree with blank bark samples affixed to it were equidistant from the source of the attraction created by the initial attacks. Therefore, it would be doubtful that the presence or absence of attack on these two trees would have resulted from a differential influence created by their position from the attraction source. The abrupt increase from 1.43 to 21.66 attacks per sq. ft. preceded by only a one-day preattack period, was probably due to the tree's proximity to the highly attractive location created by the attacks on the other two trees.

Two unique aspects of this experiment were that the first attacks were recorded on the day that the experiment was initiated and the intensity of attack was extremely high for one 24-hour period. On two of the three trees, 21.66 and 18.26 attacks per sq. ft. were recorded, each in a 24-hour period. These two factors together indicate the presence of a very high population level at the time of this test.

Exposure of phloem. The male, upon initiating an attack, penetrates the bark into the phloem and commences immediately to construct a "nuptial chamber." During this activity a small area of the phloem and the outermost layer of the sapwood is exposed. To test for the possible creation of an attraction by the exposed phloem, a paired tree experiment was initiated. Twenty areas of phloem were periodically exposed along the bole of one tree by removing one-square-inch sections of bark with a wood chisel. The attack pattern on both trees was similar in most respects and did not differ in the number of attacks received. After each exposure of phloem no abrupt rise in attack intensity was recorded which could compare to the intensity recorded on logs where excised bark samples containing initial attacks had been secured (Table III and Fig. 3). Furthermore, in all tests where excised bark samples containing initial attacks were utilized, the ensuing attack occurred before 24 hours had elapsed (Table III and Fig. 3). In this experiment, however, the control was attacked on the third day and the treated tree on the fourth day. It was concluded from these data that the exposure of phloem, independent of other factors, was not creating an attraction.

Removal of males from initial attacks. Twenty bark samples which had been attacked within the last 24 hours were cut from another source. The male was removed from the entrance tunnel by carefully flaking off enough bark to permit grasping with a forceps. If a female was encountered, she also was removed. It had been observed that the female on occasion will join the male before completion of the "nuptial chamber." However, the majority of females join the male from 24 to 48 hours after the male initiates a gallery (10).

The twenty excised, unoccupied galleries were then tested as previously described. No attacks were recorded during the course of the ten-day test on either the tree with the unoccupied galleries or the tree to which bark sections had not been attached.

Differential attraction associated with sex. An experiment was designed to evaluate the relationship between mass attraction and the sex of the beetle initiating the attack. Recently emerged adults were separated according to the described secondary sex character (5, 12) and introduced into separate cut log samples of *P. ponderosa*. The adult was secured over an artificially preformed opening in the bark with a gelatin capsule (12) by inserting insect pins through the capsule into the bark. Twenty-four hours later the capsules were removed and one-inch-square plastic screens were glued over each entrance tunnel. This technique prevented any adults in nature from joining these galleries. Thirty of these forced attacks of each sex were then excised and attached to separate felled trees positioned at a ten-foot interval.

On the tree to which bark sections containing male beetles had been

TABLE VI

RELATIVE ATTRACTION CREATED BY FORCED MALE AND FEMALE ATTACKS

Days after initiation	Number of attacks per sq. ft. of bark surface on trees with excised bark samples containing	
	Males	Females
1	1.85	0
2	3.90	0.78
3	2.21	2.11
4	0.96	1.81
5	1.47	2.14
6	0.96	0.81

attached, the greatest attack intensity occurred during the first three days following initiation of the experiment and during the third, fourth, and fifth days on the tree where bark sections containing females had been placed (Table VI). Two days after the start of the test, 5.75 attacks per sq. ft. had been recorded on the tree with male infested bark samples while less than 1 attack per sq. ft. was counted on the tree with female infested bark samples. The pattern exhibited in this test very closely parallels previous experiments in which bark samples containing only 24-hour initial attacks were fixed to one tree and the control was left blank (Table III). Actually the attack pattern created by the forced female attacks did not differ notably from the pattern exhibited on the blank controls in previous tests.

If both the male and female infested trees were equally attractive, the pattern, at least initially, would have been similar to that recorded in the "delay of the mass attack" experiment where initial attacks were affixed to both trees (Fig. 3). Both trees in this case were attacked at the same time and with the same intensity. This strongly indicates that the attraction created by initial attacks is very intimately associated with the adult male. A large number of males of the local population are responding in a very short period of time to the presence of the males initiating the first strikes.

Attraction associated with adult male maturity. If the mass attraction is specific to sex, then immature males possibly would not be expected to create the same attack pattern shown in Table VI. A test was performed in which recently transformed adult males were removed from beneath the bark and secured over preformed openings in the bark of a recently felled tree. Within 24 hours many of these teneral adults had penetrated into the phloem. At the same time, males which had recently emerged from infested logs were introduced into four felled trees arranged in a circular design with the fifth tree. After 24 hours had elapsed, the capsules which had enclosed the beetles were removed from the five trees and a daily record of the ensuing attacks was maintained.

The tree preattacked with teneral adults was unattacked throughout the 13-day test, while the remaining four control trees were attacked first on the second day and each subsequent day of the experiment. The data indicate that recently transformed adult males which had been removed from beneath the bark were not creating an attraction.

DISCUSSION

The results of the experiments reported in this study support the conclusion that the successful initial attacks of *Ips confusus* create a strong attraction which is expressed by an abrupt increase in the attack intensity (Fig. 1). This increase in the number of attacks per sq. ft. of bark surface has been termed the mass attack. Although the magnitude and the time of occurrence of this mass attack varies between experiments, its presence was generally recorded. Manipulation of the initial attack has shown conclusively that this mass influx of beetles is not a chance event and that the attraction produced is associated with the successful pioneer attacks. These data support that part of the host selection theory advanced by Person (7) which states, " . . . after a few attacks [by *Dendroctonus brevicomis*] are made a second, stronger attraction is started. . . . This secondary attraction is probably strong enough to attract beetles for a considerable distance, with the result that the tree is usually heavily attacked and killed." In addition, the results of this study corroborate the observations of Anderson (1) who studied the host selection behavior of *Ips pini*.

The pattern of attack recorded in these experiments suggests an expression of the population level at the advent of the test and in the particular locality of the test in the forest. This is indicated by the variation in the magnitude of the increase at the onset of the mass attack and the highest attack intensity recorded in a 24-hour period. On the trees where excised bark samples containing initial attacks were placed, the following increases expressed in attacks per sq. ft. were recorded: 4.40, 0.85, 0.59 (Table III), 8.97 (Table V), and 2.05 (Table VI); the highest attack intensities recorded on these trees in a 24-hour period were: 6.69, 2.02, 0.67, 10.64, and 3.90, respectively. In an experimental area where relatively uniform weather conditions prevail throughout the summer, this technique could be utilized for estimating the relative local population levels of *I. confusus*, provided a reliable sampling method could be developed. Additionally, it would appear to have further application in population dynamics studies where the frequency and magnitude of population fluctuations are quantities which must be evaluated. Future research efforts in this area may in time produce a method whereby a reliable evaluation can be made of the impact of present bark beetle control methods on subsequent population levels.

In attempting to isolate the source of attraction produced by initial attacks, exposure of the sapwood and the phloem independently did not appear to influence attractiveness. However, the sex of the beetle causing the initial attack evoked a differential response from the attacking population. With polygamous bark beetle species, typified by *I. confusus*, the male initiates the attack by excavating the entrance tunnel on the new host (3). In a previous study of this species (12), evidence supported the following conclusions regarding the influence of beetle sex on attraction: 1) females were attracted to galleries occupied only by males; 2) females were not attracted to galleries containing only females; and 3) males were not attracted to female-occupied galleries. The results from this study indicate again (Table VI) that the males of the attacking population were not responding to the presence of the female in the preattacked tree. However, the most significant result of this experiment was the positive attraction which was created by the forced male attacks (Table VI). It becomes increasingly apparent that the attraction produced by initial attack is associated with the individual male initiating these attacks. Earlier observations (12) have revealed that attacks less than 24 hours old contain predominantly males.

Past investigations [see reviews by Callaham and Shifrine (2) and Miller and Keen (6)] have been based on Person's (7) theory, i.e., the secondary (mass) attraction results from fermentation processes in the phloem produced by yeasts introduced by pioneer beetles. The fact that forced male attacks were highly attractive as compared to forced female attacks (Table VI) would not support this concept unless there exists a sex-specific association with such microorganisms. However, evidence is lacking which would support the presence of such an association. In addition, teneral adult males introduced into new host material created no attraction. These recently transformed adults should have the same opportunity to transmit the same organisms from the brood logs to the new host as newly emerged adults.

When the adults were removed from newly initiated attacks, the unoccupied galleries did not create any further attraction. Again, if the independent activity of microorganisms were responsible for the creation of the secondary attraction, subsequent attacks would have been recorded. These adults had the same opportunity to inoculate the phloem with the various associated organisms as did the adults which were not removed from their galleries (Tables III, V, and VI, and Fig. 3).

If males occupying the initial galleries are responsible independently for the attraction of males from the local population, then it should be possible to duplicate this event utilizing these males independent of their galeries. However, tests in which recently emerged adults were crushed and spread on the bark of a felled tree did not produce an attraction. Also, an attraction was not observed in caged tree (living) experiments where males and females did not initiate attacks. Anderson (1) caged crushed beetles and live beetles alone but his results were also negative.

Evidence presented in this study indicates that the secondary attraction created by initial attack probably is the result of a complex of factors which, nevertheless, is intimately associated with the presence of the live male beetle in the phloem tissue of suitable breeding material. Both males and females exhibit a positive response to the presence of the males within the initial galleries. In future studies it will be necessary to investigate the exact nature of these behavior patterns. Many studies have been predicated on the dominance of only one factor, for example, the chemotactic (primarily olfactory) response, i.e., investigations involving the production of volatile materials by yeasts (4, 7; and footnotes[5,6, and 9]). However, the intricacies of other stimulus-response patterns and their "sequence" appear to have a significant bearing on this problem and warrant further investigation.

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COMPARATIVE BIOCHEMISTRY OF OBLIGATELY PARASITIC AND SAPROPHYTIC FUNGI. I. ASSIMILATION OF C^{14}-LABELED SUBSTRATES BY NONGERMINATING SPORES[1]

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SUMMARY

Comparisons were made of the incorporation of carbon from acetate-2-C^{14} and L-leucine-U-C^{14} into CO_2, amino acids, organic acids, sugars, nucleic acids, and protein of uredospores of *Uromyces phaseoli*, *Puccinia graminis tritici*, race 56, and *Puccinia helianthi;* conidia of *Glomerella cingulata*, *Aspergillus niger*, and *Neurospora sitophila;* and chlamydospores of *Ustilago maydis*. Nongerminating uredospores utilized exogenous acetate and synthesized amino acids at a much slower rate than did conidia of the saprophytic fungi, and they rapidly lost the ability to incorporate carbon from acetate into protein. Uredospores of *U. phaseoli* incorporated L-leucine-U-C^{14} into protein to about the same extent as did conidia of *A. niger* and chlamydospores of *U. maydis*, showing that their ability to incorporate amino acids into proteins is equal to that of spores of some saprophytic fungi.

INTRODUCTION

Uredospores of rust fungi germinate readily in a favorable environment, but mycelium will not develop in culture. This suggests that these parasites may be unable to synthesize some important metabolites which they derive from their hosts. If this assumption is correct, imbalances in metabolic patterns may occur because of the failure of some essential biosynthetic reactions to proceed rapidly or at all. Therefore, it should be possible to define the symptomatology of obligate parasitism by comparing the biochemical capacities of the rust fungi with those of saprophytic species.

In this present study, nongerminated uredospores of various rusts were compared to vegetative spores of other species of fungi to determine if significant differences between the two groups occurred with respect to utilization of exogenous substrates. Radiocarbon was supplied to the spores in 2 forms: as acetate-2-C^{14} to test their capacities for synthesizing Krebs cycle and amino acids, and as L-leucine-U-C^{14} and L-methionine-S^{35} to determine how rapidly the various spores could incorporate preformed amino acids into proteins. Since these were nongerminating spores, no net synthesis of protein occurred. A preliminary report on the data herein has appeared elsewhere (16).

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MATERIALS AND METHODS

Uredospores of *Uromyces phaseoli* (Pers.) Wint., *Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn., race 56[2], and *Puccinia helianthi* Schw. were obtained from susceptible greenhouse-grown plants. Conidia of *Glomerella cingulata* (Stone.) Spauld. & von Schr. and *Aspergillus niger* van Tiegh were obtained from cultures on potato-dextrose-agar slants kept at 21° C. in the dark, employing the methods of McCallan and Wilcoxon (7). Conidia of *Neurospora sitophila* (Mont.) Shear & Dodge were dislodged from mycelia growing on Haurowitz's medium as described by Owens (10). Chlamydospores of *Ustilago maydis* (D. C.) Cda. were obtained from galls on field-grown corn. All spores used were capable of at least 70 per cent germination.

Except where noted, the spores were suspended in 0.1 per cent Tween 20 in water, centrifuged, and the supernatant solution was discarded. This treatment was repeated twice, and the spores washed once with water.

Sodium acetate-2-C^{14} was purchased from New England Nuclear Corporation, Boston, Mass., and had a specific activity of 2 millicuries per millimole. L-Leucine-U-C^{14} and L-methionine-S^{35} were biosynthesized by Schwarz Bioresearch, Inc., Mt. Vernon, N. Y. They had specific activities of 1.1 μc./mg. and 5 μc./mg., respectively. Isotopes were stored and used in water solution at a concentration of 5 μc. per ml.

Titre of spore suspensions. Sufficient spores were used to yield about 2.5 mg. of protein nitrogen, and experiments were usually carried out in a total volume of 100 ml. Replicate experiments were in closest agreement if the amount of spores in this volume was held to titres sufficient to yield between 1 and 5 mg. of protein nitrogen. To obtain 2.5 mg. of protein nitrogen, about 100 mg. fresh weight of spores were used from *Uromyces phaseoli*, *Puccinia graminis tritici*, *Puccinia helianthi*, and *Ustilago maydis*, and 1.4×10^9, 6.0×10^9, and 0.7×10^9 conidia of *G. cingulata*, *A. niger*, and *N. sitophila*, respectively. Spores were counted by the method of McCallan and Wilcoxon (7).

Incubation of spores with labeled substrates. After suspension of the spores and addition of isotope, the solutions were agitated on a flatbed shaker. When carbon dioxide was to be collected, CO_2-free air was bubbled through the mass and the expired CO_2 was trapped in $1N$ NaOH. At termination of the experiment, the spore mixture was poured into 2 volumes of boiling absolute alcohol and the solution boiled gently on a hot plate for several minutes.

Extraction. After the above treatment, the spore suspension was centrifuged and the supernatant set aside. Spores were extracted three times with 80 per cent ethanol and 3 times with hot water in a boiling water bath (17) and the supernatants combined. The spores were next extracted twice with hot 5 per cent trichloroacetic acid for nucleic acids (13, pp. 13–14). The spore residue was refluxed 16 hours with $6N$ HCl to hydrolyze protein and the residue filtered and set aside for counting[3]. Nitrogen in the protein fraction was determined by the micro Kjeldahl procedure (9). Ammonia in this fraction was determined without digestion and using $10N$ NaOH for distillation.

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[2] Obtained through the courtesy of Dr. C. E. Minarik, Fort Detrick, Frederick, Maryland.

The extracts were passed through a 10-cm. column of hydrogen-form Dowex 50-X4, 200–400 mesh, to remove the free amino acids. These were recovered from the column as described by Plaisted (11). The effluent from the Dowex 50 column was next passed through a 10-cm. column of acetate-form Dowex 1-X8, 200–400 mesh. The effluent contained nonionic materials including sugars. The nonvolatile organic acids were removed from the column with 100 ml. of $6N$ formic acid and dried in an air stream.

Chromatography and autoradiography. Both free and protein amino acids were chromatographed as described by Porter, Margolis, and Sharp (12). For quantitative analyses, duplicate samples at two different levels of concentration were chromatographed. Whatman No. 1 paper was employed for autoradiography. After drying, Kodak No-Screen X-ray film was exposed to the papers for 1 month. Three different levels of concentration were employed and the best chromatogram was used for radioactivity determinations.

Radioactivity determinations. Total activity in each fraction was determined by counting infinitely thin samples on aluminum planchets with a Nuclear-Chicago D-47 gas-flow counter fitted with a micro-mil window. Activity on the planchets was held to within 500 and 1,000 c.p.m. and all results are based on protein N[3]. For determination of activity on paper chromatograms after autoradiography, a gas-flow detector similar to that described by Fuller (3) was used.

Carbon dioxide determinations. Carbon dioxide was trapped in $1N$ NaOH and then precipitated with saturated $BaCl_2$. The $BaCO_3$ was removed by centrifugation and the excess base was determined by titration with $0.1N$ HCl with phenolphthalein as the indicator. The $BaCO_3$ was suspended in 100 ml. of absolute ethanol and an aliquot, usually 1 ml., was taken for counting.

Manometry. Respiratory measurements were made in Warburg respirometers at 30° C. by standard techniques (18). Usually 35 mg. of uredospores were suspended in 1 ml. of $0.2M$ phosphate buffer and the sys-

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[3] Separate determinations by methods similar to those of Owens (10) have shown that chitin contributes about 3 per cent of the nitrogen in the protein fractions obtained from the fungus spores studied. All the spores were similar in this respect.

TABLE I

OXYGEN CONSUMPTION BY SPORES OF VARIOUS FUNGI SUPPLIED WITH SODIUM ACETATE AT pH 6

Species	μL. O_2 consumed per mg. protein N in one hour	
	0.1 *M* Acetate	Water
Neurospora sitophila	950	550
Ustilago maydis	380	240
Aspergillus niger	280	200
Uromyces phaseoli	190	150

tem was brought to a final volume of 2.5 ml. by addition of appropriate amounts of substrate solutions.

RESULTS

As a first step in comparing the metabolism of obligately parasitic fungi with that of saprophytes, the capacities of some nongerminating fungus spores were determined for assimilating acetate and leucine. These capacities were characterized in terms of: (a) the over-all assimilation of a substrate as exemplified by ammonium acetate-2-C^{14}, (b) the extent of conversion of this material to free amino acids and protein amino acids, and (c) the incorporation of the preformed amino acids methionine and leucine into protein.

TABLE II

DISTRIBUTION OF C^{14} IN VARIOUS COMPONENTS OF SPORES AFTER INCUBATION FOR 5 HOURS IN 0.02 *M* $C^{14}H_3COONH_4$

Fraction	Distribution of C^{14} as per cent of total						
	Uromyces phaseoli	*Puccinia graminis tritici*	*Puccinia helianthi*	*Ustilago maydis*	*Glomerella cingulata*	*Aspergillus niger*	*Neurospora sitophila*
Amino acids	12	22	16	24	38	29	17
Organic acid	38	34	42	37	8	17	12
Sugars	26	25	26	21	7	32	34
Protein	9	12	8	13	37	17	27
Nucleic acids	9	6	7	4	10	4	9
Residue	6	1	1	1	Trace	Trace	Trace
C.p.m. assimilated per mg. protein N	4,300	8,500	7,300	18,300	171,000	96,200	288,000
C.p.m. emitted as $C^{14}O_2$ per mg. protein N	3,200	5,200	7,600	11,300	51,600	36,500	103,500
Specific activity CO_2 (c.p.m. per μ*M* carbon)	90	80	130	200	290	190	540
Weight N in protein (mg.)							
Initial	3.9	1.6	1.5	0.9	1.1	0.8	1.4
Final	3.3	1.5	1.5	1.1	2.3	0.6	1.6
Weight NH_3 in protein (mg.)							
Initial	0.5	0.2	0.2	0.2	0.2	0.1	0.1
Final	0.5	0.3	0.2	0.2	0.4	0.2	0.3

GENERAL UTILIZATION OF ACETATE

Oxygen consumption. The relative consumption of oxygen by the spores of 4 fungi in the presence and absence of acetate is shown in Table I. Conidia of *N. sitophila* were most active, whereas uredospores of *U. phaseoli* were least active. Acetate increased oxygen consumption of all spores and the relative increase was roughly proportional to relative endogenous respiratory activity of each species. Ammonium acetate was later found to be as satisfactory as sodium acetate for all spores and was used in subsequent experiments.

TABLE III

TYPICAL MOLAR COMPOSITION OF FREE AND PROTEIN AMINO ACID FRACTIONS FROM FUNGUS SPORES

Amino acid	Micromoles recovered per mg. protein N as % of total			
	Free amino acids		Protein amino acids	
	Uromyces phaseoli	*Aspergillus niger*	*Uromyces phaseoli*	*Aspergillus niger*
α-Alanine	2	3	9	11
β-Alanine	Trace	0	—	—
γ-Aminobutyrate	Trace	0	—	—
Arginine	10	2	4	5
Asparagine	1	Trace	—	—
Aspartate	7	5	2	5
Glutamate	15	23	11	10
Glutamine	21	43	—	—
Glycine	4	3	9	9
Histidine	7	Trace	Trace	Trace
Leucine(s)	3	5	17	16
Lysine	7	6	8	7
Methionine sulfoxide	Trace	Trace	5	2
Phenylalanine	5	6	6	7
Proline	—	—	9	5
Serine	2	3	6	9
Threonine	4	2	4	6
Tyrosine	5	4	4	4
Valine	9	2	5	5
Micromoles per mg. protein N	2.7	1.8	46	24

Assimilation of $C^{14}H_3$ $COONH_4$. Table II summarizes a comparative study in which spores of the various fungi were incubated for 5 hours with 0.02M ammonium acetate-2-C^{14}. The rusts assimilated only about 5 per cent as much acetate as did the saprophytes and less than half as much as did *U. maydis.* The rusts and smuts showed more nearly similar patterns of acetate utilization than did less parasitic fungi. Their organic acid fractions were more heavily labeled and both accumulated very little radioactivity in protein. Spores of *G. cingulata* and *N. sitophila* were especially active, with highly labeled protein fractions. The data showed that there was little increase in protein N except in spores of *G. cingulata*. Thus, incorporation of label into protein of all species, except *G. cingulata*, was accomplished without net protein synthesis.

The production of CO_2 by the rusts and smuts was substantially the same, averaging about 2.5 mg. of CO_2 per mg. protein nitrogen. The saprophytic fungi averaged 8.7 mg. of CO_2 per mg. protein nitrogen. The specific activity of the CO_2 produced by spores of the saprophytic fungi did not greatly exceed that of *P. helianthi*, except for conidia of *N. sitophila*, and exceeded that of *U. phaseoli* and *P. graminis tritici* by a factor of only about two to three. The specific activity of the CO_2 produced by conidia of *N. sitophila* was only one-third that of the added acetate on a molar carbon basis, showing that most of the carbon dioxide was derived from endogenous sources in all the fungi.

The compositions of the amino acid and protein fractions of spores of *A. niger* and *U. phaseoli* are shown in Table III. In general, the compositions of the protein fractions were similar, with the leucines predominating. The compositions of the free amino acid fractions from the two types of spores were also quite similar.

AMINO ACID SYNTHESIS FROM ACETATE

Protein amino acids. The distribution of radiocarbon among protein amino acids after exposure of the spores to radioacetate is shown in Table IV. Taking the 3 species of rust fungi together, after 2 hours, radioactivity appeared only in alanine, aspartate, and glutamate, while lysine was found to be labeled also in *P. helianthi*. The other fungi showed a much wider distribution of C^{14}, most of it being contained in alanine, aspartate, glutamate, and the leucines. None of the fungi labeled glycine, histidine, or methionine sulfoxide, indicating a negligible synthesis of these from the methyl carbon of acetate by this group of fungi.

When these studies were extended for two of these fungi to 10 and 24 hours, spores of *U. phaseoli* were found to have synthesized a greater number of amino acids (Table VI). As before, most of the radioactivity was in glutamate. Phenylalanine, histidine, and methionine sulfoxide were not synthesized by spores of either species, and glycine and tyrosine were labeled only slightly after 24 hours by spores of *A. niger*. *U. phaseoli* did not incorporate valine or the leucines into protein after their synthesis from acetate.

The specific activities of the protein amino acids were determined for *A. niger* and *U. phaseoli* at 2-, 10-, and 24-hour periods. These specific activities are shown in Table V. During the first 2 hours, aspartate had the highest specific activity in both spores, followed by glutamate and alanine. The main differences between the spores lay in the slow labeling and lower

TABLE IV

DISTRIBUTION OF C^{14} AMONG AMINO ACIDS FROM FUNGUS SPORES INCUBATED 2 HOURS WITH 10 μC. $C^{14}H_3COONa$

Amino acid	Distribution of C^{14} as per cent in different species											
	Uromyces phaseoli		*Puccinia graminis tritici*		*Puccinia helianthi*		*Ustilago maydis*		*Aspergillis niger*		*Neurospora sitophila*	
	Free	Protein	Free	Protein	Free	Protein	Free	Protein	Free	Protein	Free	Protein
Alanine	2	19	3	50	2	7	2	10	1	14	8	8
γ-Aminobutyrate	Trace	—	0	—	1	—	0	—	1	—	Trace	—
Arginine	—	0	—	0	—	0	—	3	—	3	—	0
Asparagine	1	—	0	—	3	—	Trace	—	0	—	1	—
Aspartate	9	30	Trace	0	10	9	4	26	6	25	12	24
Cysteate	0	0	0	0	0	0	Trace	11	2	0	0	4
Glutamate	15	43	12	50	12	81	24	17	25	26	22	28
Glutamine	65	—	51	—	68	—	62	—	60	—	51	—
Glycine	0	0	0	0	0	0	0	0	0	0	0	0
Histidine	Trace	0	4	0	0	0	0	0	0	0	0	0
Leucine(s)	0	0	0	0	0	0	1	14	1	6	4	19
Lysine	0	0	1	0	0	2	1	6	Trace	8	0	0
Methionine sulfoxide	0	—	0	—	0	—	0	—	0	—	0	—
Phenylalanine	0	0	0	0	0	0	0	0	0	0	0	4
Proline	—	0	—	0	—	0	—	2	—	3	—	1
Serine	1	0	Trace	0	0	0	1	0	1	9	1	0
Threonine	Trace	0	0	0	2	0	1	3	Trace	2	0	0
Tyrosine	0	0	0	0	0	0	0	2	0	0	0	4
Valine	0	0	22	0	0	0	0	5	0	3	0	6
Unknown	4	0	5	0	1	0	2	0	1	0	0	0
Total c.p.m. per mg. protein N	29,000	2,200	25,800	1,000	28,400	3,600	111,000	52,800	1,280,000	116,000	36,400	39,400

TABLE V

SPECIFIC ACTIVITY OF AMINO ACIDS FROM FUNGUS SPORES AFTER INCUBATION WITH 10 μC. $C^{14}H_3COONa$

Amino acid	C.p.m. per μM acid in different species after various hours of exposure											
	Uromyces phaseoli						*Aspergillus niger*					
	2		10		24		2		10		24	
	Free	Protein	Free	Protein	Free	Protein	Free	Protein	Free	Protein	Free	Protein
Alanine	11,600	100	29,000	400	12,000	500	256,000	6,350	53,000	5,100	65,400	7,500
γ-Aminobutyrate	Trace	—	Trace	—	0	—	—	—	0	—	0	—
Arginine	0	0	5,400	440	3,300	180	0	2,900	0	1,840	0	2,030
Asparagine	9,700	—	48,000	—	Trace	—	0	—	0	—	0	—
Aspartate	13,700	717	49,500	8,350	4,737	10,200	853,000	24,200	117,800	42,400	36,300	46,700
Cysteate	0	0	0	0	0	0	—	0	0	0	0	0
Glutamate	10,900	185	36,200	2,200	24,750	2,700	780,500	12,600	297,300	41,400	103,700	44,700
Glutamine	18,300	—	50,800	—	25,789	—	49,900	—	103,200	—	58,000	—
Glycine	0	0	0	0	0	0	Trace	0	0	0	0	1,110
Histidine	Trace	0	0	0	0	0	0	0	0	0	0	0
Leucine(s)	0	0	9,050	0	3,750	0	142,200	1,830	559,400	2,900	169,500	1,280
Lysine	0	0	3,800	790	Trace	1,320	Trace	5,460	0	14,300	0	8,600
Methionine sulfoxide	0	0	Trace	0	Trace	0	0	0	0	0	0	0
Phenylalanine	0	0	0	0	0	0	0	0	0	0	0	0
Proline	—	0	—	65	—	260	—	2,900	-	5,500	—	8,100
Serine	5,800	0	4,300	470	12,000	370	256,000	4,700	0	3,000	0	3,300
Threonine	Trace	0	6,000	150	5,000	580	Trace	1,660	0	1,580	0	5,200
Tyrosine	0	0	0	0	0	0	0	0	0	0	0	2,400
Valine	0	0	39,200	0	0	0	0	2,900	0	1,840	0	6,100

specific activities of protein amino acids. Even spores of *U. maydis*, which were closest to the rust fungi in magnitude of substrate incorporation, rapidly synthesized 11 amino acids compared to the 4 synthesized by spores of *P. helianthi* (Table IV).

Upon extension of these studies to 10 and 24 hours, it was found that the specific activities of most of the amino acids had ceased to increase or had declined (Table V). In general, the pattern of specific activity of amino acids was similar in spores of all species at each time interval.

Free amino acids and amides. The incorporation of radioacetate into free amino acids and amides after 2 hours was undertaken to complete the study of amino acid synthesis by fungus spores. These studies were extended to 10 and 24 hours in the case of *U. phaseoli* and *A. niger* spores.

Figure 1 shows that C^{14} from acetate was incorporated into the amino acid fraction of *A. niger* very rapidly, reaching a maximum within 1 hour after addition of acetate. By 3 hours the level of radioactivity fell, corresponding to the time when germination of the spores would have begun. In contrast to this, radioactivity was incorporated slowly in *U. phaseoli*, reaching a maximum by 4 hours after a lag of a half hour.

A study of the distribution of C^{14} among the free amino acids and amides after 2 hours with acetate (Table IV) revealed almost the same distribution of radioactive acids in the rust and saprophytic fungi. None of the spores synthesized phenylalanine, glycine, methionine sulfoxide, or tyrosine from acetate. In spores of all of the species, glutamate and glutamine contained most of the labeled carbon. Uredospores of *P. graminis tritici* contained radioactive valine which was not found among protein amino acids.

The specific activities of the free amino acids and amides from spores of *U. phaseoli* and *A. niger* are given in Table V. During the first 2 hours, glutamine had the highest specific activity in spores of *U. phaseoli*, while aspartate, closely followed by glutamate, was not highly labeled in *A. niger*. After 24 hours, spores of both species had lost radioactivity from their free amino acid fractions compared to 10 hours, and the pattern of radioactivity found among the amino acids at 24 hours reflects this (Table VI). Thus, valine was very radioactive in *U. phaseoli* by 10 hours, but not after 24 hours. Total radioactivity in the free amino acid fraction reached a peak by 2 hours in *A. niger*, but not until 5 hours in *U. phaseoli*. Both the leucines and valine were synthesized from acetate in 10 hours by *U. phaseoli*, but were not incorporated into protein even after 24 hours although the amount of radioactivity increased continuously in this fraction.

The specific activities of the various free amino acids at 10 and 24 hours are given in Table V. In conidia of *A. niger*, the leucines had the highest specific activity, followed by glutamate. Valine was not labeled. At

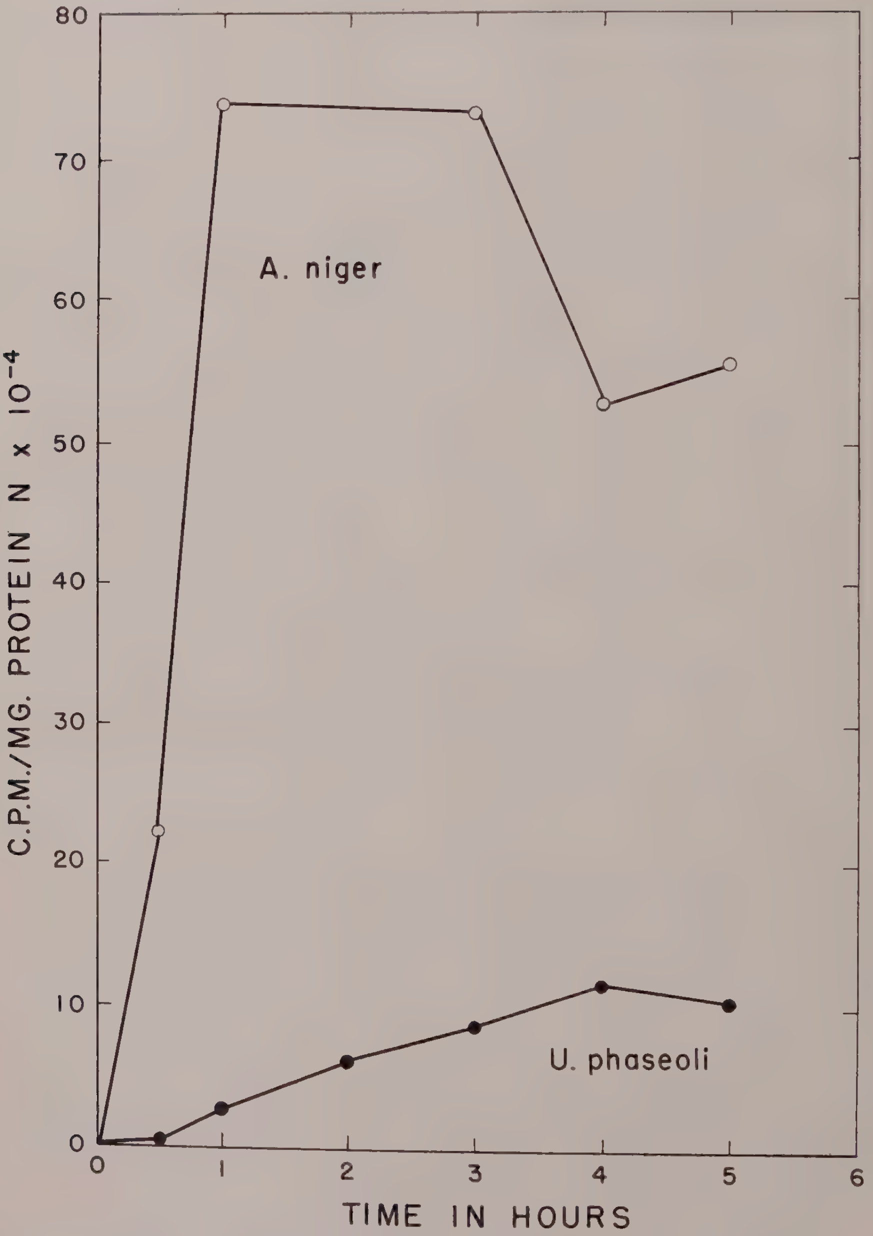

FIGURE 1. Amino acid formation by fungus spores from acetate-2-C^{14}.

TABLE VI

DISTRIBUTION OF C^{14} AMONG AMINO ACIDS FROM FUNGUS SPORES INCUBATED WITH 10 μc. $C^{14}H_3COONa$

Amino acid	Distribution of C^{14} as per cent in different species after various hours of exposure							
	Uromyces phaseoli				*Aspergillus niger*			
	10		24		10		24	
	Free	Protein	Free	Protein	Free	Protein	Free	Protein
Alanine	2	6	4	6	1	6	3	8
γ-Aminobutyrate	1	—	0	—	0	—	0	—
Arginine	2	3	2	4	0	1	0	2
Asparagine	2	—	Trace	—	0	—	0	—
Aspartate	13	29	3	27	4	23	3	23
Cysteate	—	—	—	—	—	—	—	—
Glutamate	20	43	33	40	46	45	39	44
Glutamine	40	—	50	—	28	—	39	—
Glycine	0	0	0	0	0	0	0	1
Histidine	0	0	0	0	0	0	0	0
Leucine(s)	1	0	1	0	19	5	14	2
Lysine	1	11	Trace	14	0	11	0	6
Methionine sulfoxide	1	0	Trace	0	0	0	0	0
Phenylalanine	0	0	0	0	0	0	0	0
Proline	—	1	—	3	—	3	—	4
Serine	1	5	2	3	0	3	0	3
Threonine	1	1	2	3	0	1	0	3
Tyrosine	0	0	0	0	0	0	0	1
Valine	13	0	0	0	0	1	0	3
Total c.p.m. per mg. protein N	72,000	26,500	30,000	35,000	265,000	221,000	109,000	244,000

10 hours, valine had a higher specific activity than glutamate in *U. phaseoli*.

Rate of label incorporation into the protein fraction. When spores were harvested at hourly intervals from a suspension containing sodium acetate-2-C^{14}, it was observed that C^{14} in the protein from *U. phaseoli* and *A. niger* increased continuously, whereas it at first increased in free amino acids and then declined. When radioactivity in the protein fractions was plotted against the logarithm of time after wetting the spores, linear relationships were obtained for both fungus species (Fig. 2). The regression equation relating C^{14} assimilated, in terms of c.p.m. $\times 10^{-3}$ per mg. protein nitrogen (L), to time of incubation in hours (t) was found to be

$$L = 26 \log_{10} t - 0.8 \qquad (1)$$

from uredospores of *U. phaseoli*, with a correlation coefficient for +0.975. For conidia of *A. niger* the regression equation was found to be

$$L = 220 \log_{10} t + 3.7 \qquad (2)$$

with a correlation coefficient of =0.995. Thus it is seen that the regression coefficient relating activity in the protein fraction to the logarithm of incubation time was 8.6 times higher for spores of *A. niger* than for those of *U. phaseoli*, suggesting that the latter species suffers a rapid loss of the mechanism for protein synthesis after being wetted. This loss, coupled with slow synthesis of valine and leucine, could account for the failure to detect label in valine and leucine in the protein fraction.

THE LABELING OF PROTEIN FROM EXOGENOUS L-LEUCINE-U-C^{14} AND L-METHIONINE-S^{35}

An alternative to the above, that leucine could not be incorporated into protein, was tested by using L-leucine-U-C^{14}. L-Methionine-S^{35} was included as a representative of those amino acids not synthesized from acetate. The ready incorporation of these exogenous amino acids was demonstrated to occur as for any other tissue (1, 13).

L-Leucine-U-C^{14}. In the first five hours after wetting spores of *U. phaseoli* and adding leucine-C^{14}, 11,000 c.p.m. per mg. protein N was found in the protein fraction, while when label was added in the second 5-hour

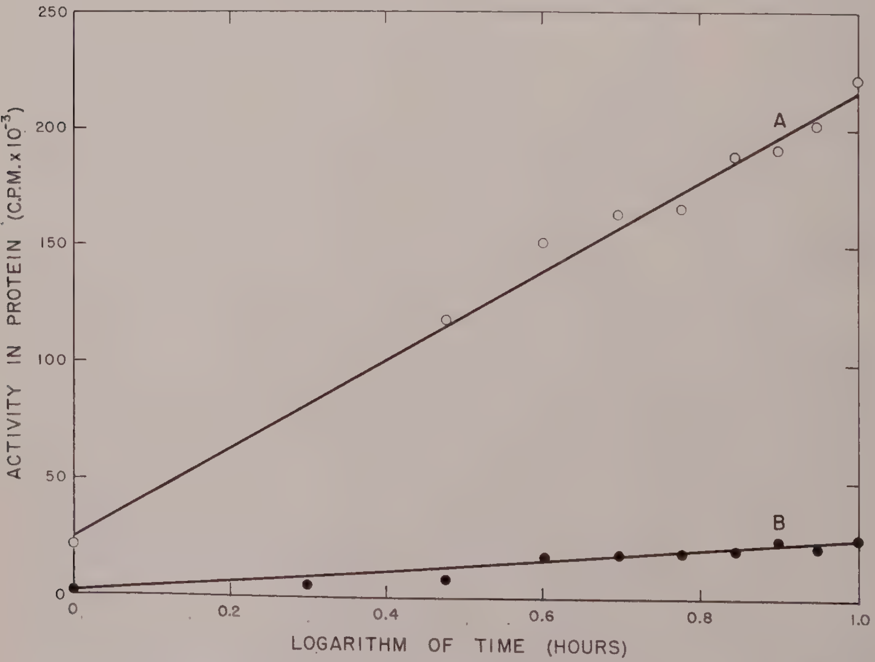

FIGURE 2. Rate of incorporation of C^{14} from $C^{14}H_3COONa$ into the protein fractions of (A) conidia of *Aspergillus niger* and (B) uredospores of *Uromyces phaseoli.*

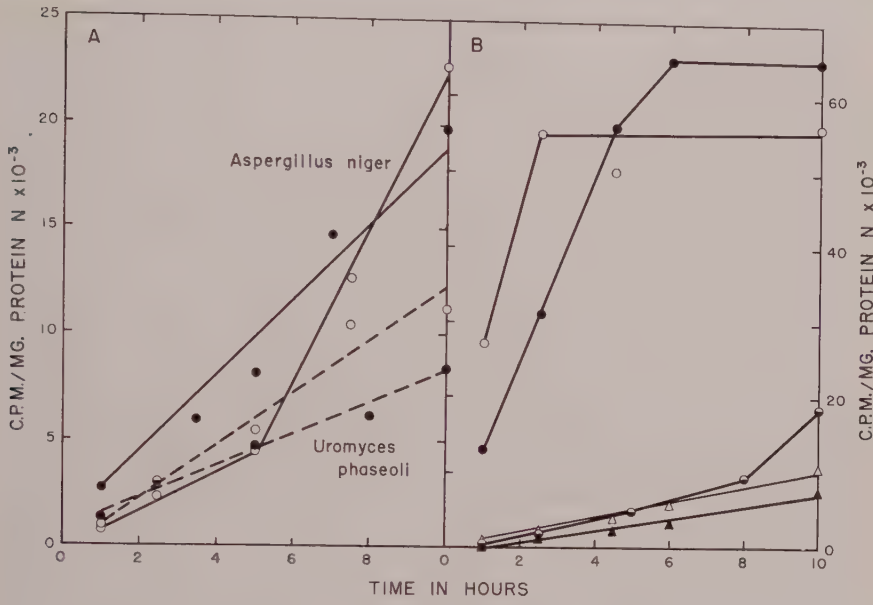

FIGURE 3. Incorporation of substrates into protein of fungus spores after different intervals. (A) With 1 μc. L-leucine-U-C^{14}. Circles, leucine alone. Filled circles, 1 per cent glucose. (Broken lines) *Uromyces phaseoli.* (Solid lines) *Aspergillus niger.* (B) With 1 μc. L-leucine-U-C^{14} and 1 per cent glucose. Filled triangles—*Puccinia graminis tritici;* triangles—*Puccinia helianthi;* half-filled circles—*Ustilago maydis;* circles—*Glomerella cingulata;* filled circles—*Neurospora sitophila.*

period (5 to 10 hours), 10,000 c.p.m. per mg. protein N was found. Only 0.5 per cent of the total available leucine was incorporated by the uredospores, half of this being converted to CO_2 in 5 hours. After 5 hours, only leucine was radioactive in protein hydrolyzates of uredospores. By 10 hours, some activity was also detected in glutamate and aspartate.

Spores of saprophytic fungi varied considerably in their utilization of leucine for protein synthesis. Conidia of *A. niger* rapidly incorporated leucine into protein, but showed no conversion of it to other amino acids even after 10 hours. Spores of *U. maydis*, *G. cingulata*, and *N. sitophila* were able to label the whole spectrum of amino acids in protein from leucine. In fact, conidia of *G. cingulata* and *N. sitophila* converted leucine to such an extent that, by 10 hours, label in leucine was only minor compared to that in many of the other amino acids.

L-Methionine-S^{35}. Results similar to those observed with leucine were obtained using methionine-S^{35}. Uredospores of *U. phaseoli* incorporated methionine into protein as they did with leucine. Some of the sulfur from methionine was found in cysteine.

PROTEIN SYNTHESIS FROM L-LEUCINE-U-C^{14}

Since there was no net synthesis of protein in the spores (Table II), incorporation of radioactive leucine was employed as a more sensitive measure of protein synthesis (4).

Uredospores of Uromyces phaseoli. Figure 3 A depicts the incorporation of leucine by uredospores of *U. phaseoli* in the presence and absence of glucose. In both circumstances the incorporation was nearly linear and of approximately the same order of magnitude.

Spores of other species. Figure 3 B depicts the incorporation of leucine carbon into the protein fractions from spores of *U. maydis*, *P. graminis tritici*, and *P. helianthi*. Both types of uredospores exhibited nearly the same trend and magnitude of label incorporation as uredospores of *U. phaseoli*. In the early stages of leucine utilization, spores of *U. maydis* and *P. helianthi* were nearly identical, but the chlamydospores finally attained about twice the radioactivity that the rust spores did. In the presence of glucose, the incorporation of leucine into *A. niger* spore proteins was linear and less adaptive in character (Fig. 3 A). For this reason, glucose was employed as an energy source when intraspecific comparisons were to be made.

From Figure 3 B, it can be seen further that spores of both *N. sitophila* and *G. cingulata* have a rapid early leucine consumption rather than the linear incorporation shown by *A. niger*. In the ungerminated condition, this rapid early uptake soon ceased, probably because growth was prevented through omission of required salts for germination (7).

DISCUSSION

Studies on the metabolic capacity of the uredospore (5, 8, 14, 17) have been difficult to assess for clues to their failure to grow beyond the germination stage in the absence of the living host. Since there are no sound criteria to recognize such clues, it would seem wise to attempt the establishment of a basis for recognition, especially in view of the fact that the ultimate explanation of obligate parasitism may prove to be merely a quantitative one (14, 17). One method would be to choose a set of fungi ranging in degree of parasitic attack from obligate to saprophytic, and to study them in concert such that the data obtained might be assessed for the quality desired, i.e., that factor in the spore which responds to host materials.

There are a number of criteria that would be desired in the selection of any set of fungi. The fungi should: abundantly sporulate on common media; not be too vigorous as regards oxygen consumption and substrate utilization; show synchronous, ready spore germination; and be broad in tolerance to the environment. Not all of these criteria have been met in choice of fungi for this study, but the search is a continuing one. It had been hoped to draw all of the facultative parasites from a host also attacked by one of the rust species, but this was not possible. At present, the most suitable spore appears to be the conidium of *A. niger*. The spores have nearly the same endogenous oxygen consumption as uredospores, exhibit approximately the same protein synthetic capacity in the ungerminated state, germinate nearly synchronously in 6 hours (19) compared to 3 for uredospores, sporulate readily on potato dextrose agar, and otherwise are rather easy to handle. This saprophyte does, however, require at least sucrose and ascorbic acid for germination[4], although orange juice is best (7). For these reasons, conidia of *A. niger* have been chosen for the most detailed studies with uredospores, usually those of *U. phaseoli*.

A number of materials increase the oxygen consumption of uredospores (2, 15, 17) and it had been assumed that this response was correlated with an increased utilization of these materials. Shu *et al.* (14) and especially Farkas and Ledingham (2) have found that while external substrates were utilized, much of the carbon dioxide released under such conditions was derived from endogenous substrates. The first step in a comparative study such as this was to determine the relative respiratory activity of the fungi used. A comparison has already been made for a number of these fungi by McCallan *et al.* (6) using 2 per cent sucrose. It was evident from our studies that manometry was a poor tool in the study of intraspecific metabolism, at least when the results were expressed on a protein nitrogen basis. For example, conidia of *A. niger* consumed only 30 per cent more oxygen in the first hour than uredospores of *U. phaseoli*, yet utilized 20 times the amount of radioacetate (Tables I, II). Spores of *U. maydis*, on the other hand, consumed 20 per cent more oxygen than uredospores, but utilized merely 4 times the amount of acetate.

As a result of feeding acetate to the ungerminated spores under comparable conditions (Table II), the first important observation to be made was the extremely low utilization of acetate by uredospores compared to saprophytic spores. Acetate consumption by saprophytic spores varied from twice to more than 40 times that of uredospores, and it would appear that the critical range of substrate consumption for growth lies between 2 and 10 times that found to be consumed by uredospores. There was a strong similarity between spores of the rusts and those of *U. maydis* in the distribution of label between the different fractions studied. The total acetate consumed by spores of *U. maydis* was little more than twice that of spores of *P. graminis tritici*. Judging from the general distribution of label, it would seem that the main difference between sluggish saprophytic growth and an obligate requirement for a living host would be a mere doubling of substrate consumed. In this respect, it was interesting that spores of a saprophyte, *A. niger*, were labeled in the protein fraction percentagewise to about the same extent as spores of *U. maydis* and *Puccinia graminis tritici*. It is apparent then that the major difference between obligate and saprophytic spores that can be deduced from these experiments was in total consumption of substrate and not in distribution of materials.

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[4] STAPLES, RICHARD C. Unpublished results.

When the fractions were studied chromatographically, a second difference emerged. Although label moved into the protein fraction of ungerminated spores of *U. phaseoli* about as rapidly as in those of *A. niger* or *U. maydis*, the label remained confined to Krebs cycle amino acids for a longer period of time in spores of *U. phaseoli*. This suggested that the rate of synthesis of amino acids remote from the Krebs cycle was slower in spores of *U. phaseoli* as compared to those of *U. maydis* or *A. niger*. The data in Figure 1 show that synthesis of free amino acids and amides was very slow in *U. phaseoli*, reaching a maximum only after 4 hours. Conidia of *A. niger* reached a maximum within 1 hour.

The failure to detect radioactivity in leucines and valine in protein from spores of *U. phaseoli* was interesting. Free valine ultimately became radioactive. While other amino acids were found that were not readily synthesized by the saprophytic fungi, valine and the leucines were synthesized and converted to protein by them. Thus the results with valine and the leucines suggested that either protein cannot be synthesized from some free amino acids by the uredospore, or their synthesis was especially slow and carbon from acetate never reached protein via these acids.

From Figure 2 it can be seen that protein synthesis from acetate increased at a logarithmically decreasing rate in ungerminated spores, and that the rate of this decrease was about 85 times faster in spores of *U. phaseoli* compared to those of *A. niger*. On the other hand, the rate of incorporation of leucine into uredospore proteins was not altered with time, being arithmetically linear (Fig. 3 A). Consequently valine and the leucine(s) were probably synthesized too slowly for their significant incorporation into protein. The leucine data show that once amino acids are formed their condensation to protein was unimpaired.

In the uredospore, leucine incorporation into protein was correlated with acetate incorporation into protein and not with total acetate consumption (Table II and Fig. 3). For example, spores of *N. sitophila* and *G. cingulata* both exhibited high leucine incorporation and high percentages of acetate conversion to protein, while those of *A. niger* and *U. maydis* were not much better than uredospores of *U. phaseoli* in conversion of substrates to protein or of leucine to protein. This lends support to the idea that incorporation of carbon into the protein fractions of the spores is not affected by the ability of the spore to make protein, but upon the power to withdraw carbon from the external medium and to convert it to precursors for protein synthesis. These rates were low in uredospores compared to saprophytic fungi.

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THE BIOCHEMICAL COMPOSITION OF COCONUT WATER (COCONUT MILK) AS RELATED TO ITS USE IN PLANT TISSUE CULTURE

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and Henry J. Laurencot, Jr.

SUMMARY

Fresh and autoclaved coconut water samples from mature market coconuts are compared for their content of free and combined amino acids, nonvolatile organic acids, sugars, and nucleic acid phosphorus. Data for coconut water from young and mature green coconuts are included.

Malic is the predominant organic acid; shikimic and quinic and two unidentified acids are present in all samples. Alanine, γ-aminobutyric acid, and glutamic acid constitute about 75 per cent of the free amino acids of the fluid from mature fresh coconuts; glutamine, arginine, asparagine, alanine, and aspartic acid make up about 70 per cent for young green coconuts. The sugar content increases from 0.9 g./100 ml. in young fruits to 2.2 g./100 ml. in mature green coconuts. The ribonucleic acid phosphorus content of fruits of different ages is fairly constant; the deoxyribonucleic acid increases with the age of the fruits. The relation of these data to the culture of plant tissues is discussed.

INTRODUCTION

Coconut water[1], the liquid endosperm of *Cocos nucifera* L., is used as a supplement in media for the growth of plant tissue cultures. Tissues from embryos, roots, stems, endosperm, and pollen of plants, including angiosperms and gymnosperms, may be grown on media containing this liquid (10). The early success of van Overbeek, Conklin, and Blakeslee (50) in stimulating the growth of young *Datura* embryos through the use of coconut water led to its widespread use as an adjuvant for nutrient media. Large volume cultures of plant cells (48) and yeasts, fruit flies, orchid seeds, and fungus parasites of animals (26) may be nourished by media containing coconut water. Investigations on the composition of coconut water are reported by Soto Pradera, Fernandez, and Calderin (38) and Baptist (1), and its growth promoting properties described by Shantz (35), Steward and Shantz (44), and others. In addition, there is much literature on the composition of coconut products such as coconut oil, copra, and coconut

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[1] The liquid endosperm is often called coconut milk; this use is probably derived from the French term, *lait de coco*. In English, the proper term is coconut water (24, 38); this reserves the name, coconut milk, to designate the white liquid obtained by grating coconut meat in water. This milk is an important food in many parts of the world (23, 24). Analysis of coconut meat by Nicholls (17) gave 35 to 45 per cent oil, 15 per cent carbohydrate, and 5 per cent protein; coconut water contains about 0.1 per cent oil, 5 per cent sugars, and 0.5 per cent protein (Pinto, 27; Platt 29). This is an additional reason for making a clear distinction between coconut water and coconut milk.

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meat; these items form a large dietary component for many people living in areas where the coconut is a major crop (13, 16, 24, 25).

The greatest amount of coconut water is found in young green coconuts. The coconut water provides nourishment for the growth of the solid endosperm (coconut meat) inside the hard shell of the fruit. At maturity, both the solid endosperm and the remaining coconut water serve as nutrients for the developing embryo and seedling. In this manner, coconut water serves as a natural reservoir of materials for tissue growth. The coconut fruit is exceptional in having large amounts of this liquid present over periods of a year or more.

The purpose of this paper is to describe the normal constituents of coconut water as it is used in the culture of plant tissues. A comparison is made of the constituents of fresh and autoclaved coconut water from mature fruits procured from the local market and between young green and mature green fruits collected from coconut trees. Analyses were made for free and combined amino acids, nonvolatile organic acids, sugars, ribonucleic acid phosphorus (RNA-P), and deoxyribonucleic acid phosphorus (DNA-P).

MATERIALS AND METHODS

Coconut water samples. The liquid from coconuts of three different ages was used for analysis. Young and mature green coconuts were collected from trees in Deerfield Beach, Florida; mature coconuts from the Dominican Republic were purchased at produce outlets in New York City. Because of the different sources of fruits used in these analyses, the best comparisons of data are between coconut water samples from young and mature green coconuts and between the fresh and autoclaved samples from market fruits.

The young coconuts were approximately 6 to 7 inches in diameter; they contained about 400 ml. of coconut water per fruit and had only a very thin layer of gelatinous endosperm. The mature green coconuts were 8 to 10 inches in diameter and had $\frac{1}{2}$ to $\frac{5}{8}$ inch of solid endosperm tissue. This stage of development is about 300 days post-pollination, according to Chandrasena (5). The market coconuts consisted of dehusked fruits which had undergone shipment and storage for 1 to 2 months. The thickness of the solid endosperm of these coconuts was $\frac{1}{2}$ to $\frac{5}{8}$ inch. The coconut water from all of these fruits was examined and any contaminated samples discarded. The coconut water from each group of fruits was combined and frozen until used for analysis.

A common procedure in making culture media containing coconut water is to use 5 to 20 per cent by volume. The medium is then sterilized by autoclaving for 20 minutes at a pressure of 15 pounds per square inch. The autoclaved coconut water discussed here was sterilized separately, apart from the medium, before analysis. The pH of the coconut water (4.6 to 5.6) did not change appreciably with autoclaving.

Analytical techniques. The coconut water was made 80 per cent ethanolic, the precipitated protein was centrifuged down and washed four times with 80 per cent ethanol at 50° C. The supernatant fluids were combined. The ethanolic extract, representing about 100 ml. of coconut water, was passed through a 1 × 10 cm. bed of hydrogen-form Dowex 50-X8, 200–400 mesh, to take up free amino acids and amides. The filtrate was passed through a 1 × 10 cm. bed of acetate-form Dowex 1-X10, 200–400 mesh, to take up nonvolatile organic acids. The filtrate contained the sugars and other neutral substances. The ethanol of this fraction was removed by reduced pressure, then freed of pigments and lipoidal substances by emulsifying with water-chloroform, centrifuging, and saving the aqueous layer. The free amino acids were eluted from Dowex-50 resin by the method of Plaisted (28) and were analyzed quantitatively (32). Organic acids were separated on Dowex-1 by gradient elution with $2.5N$ acetic acid followed by $6N$ formic acid (20). The individual acids were identified by one-dimensional paper chromatography as described by Weinstein, Porter, and Laurencot (53) and Weinstein and Laurencot (51).

The alcohol-insoluble residues were lyophilized. The total nitrogen of the alcohol-insoluble residues was determined by a semi-micro Kjeldahl procedure (19, pp. 805–806). For protein amino acids, aliquots of the residues were hydrolyzed in sealed ampules with $6N$ HCl at 15 pounds pressure for 15 hours. The hydrolyzates were filtered and taken to dryness several times on a steam bath; they were then taken up in 10 per cent isopropanol and the amino acids determined by two-dimensional paper chromatography (32).

Sugars were separated by one-dimensional descending paper chromatography using Whatman No. 3 paper and the solvent *n*-butanol—acetic acid—water (4:1:5 v/v/v). Quantitative results were obtained by the method of Steward, Bidwell, and Yemm (41), as used previously (52).

Ribonucleic acid (RNA) and deoxyribonucleic acid were extracted from the alcohol-insoluble residue by a differential perchloric acid method (12). The phosphorus of the nucleic acids was determined by a reduced molybdate procedure (31) following digestion in perchloric acid.

RESULTS

The total free amino acid content was highest in autoclaved coconut water from mature coconuts procured from the market (757.3 μg./ml.), as shown in Table I. The same coconut water, unautoclaved, contained 685.0 μg./ml., that from mature green coconuts, 565.8 and young green coconuts, 190.5 μg./ml. By taking into account the amount of fluid per coconut (see Table II), it can be calculated that the total free amino acid content

TABLE I

Free Amino Acids of Coconut Water from Fruits of Three Different Ages

Amino acids	μG./ml., average of two determinations			
	Young, green	Mature, green	Mature, fresh	Mature, autoclaved
Aspartic	11.3	35.9	5.4	11.4
Glutamic	9.4	70.8	78.7	104.9
Serine	7.3	45.3	65.8	85.0
Glycine	1.3	9.7	13.9	18.0
Asparagine	17.1	10.1	10.4	25.3
Threonine	2.9	16.2	26.3	27.4
Alanine	16.4	127.3	177.1	198.0
Glutamine	80.0	45.4	13.4	2.0
Histidine	3.5	6.3	Trace	Trace
Lysine	4.4	21.4	22.5	13.0
Arginine	14.7	25.6	16.8	20.7
Proline	4.1	31.9	21.6	12.9
Valine	5.6	20.6	15.1	15.5
Leucines	6.2	37.3	31.7	33.0
Phenylalanine	—	—	10.2	Trace
Tyrosine	0.9	6.4	3.1	Trace
γ-Aminobutyric	1.9	34.6	168.8	173.2
Hydroxyproline	Trace	4.1	Trace	8.2
Homoserine	—	—	5.2	8.8
Methionine	3.5	16.9	Trace	Trace
Total	190.5	565.8	685.0	757.3

per fruit is 145.4 mg. for autoclaved coconut water from mature market coconuts; 143.2 mg. for mature green; 131.5 mg. for mature fresh market fruits; and 76.2 mg. for young green coconuts.

The principal amino acids in coconut water from fresh market fruits are alanine, γ-aminobutyric acid, glutamic acid, and serine; together these comprise about 75 per cent of the free amino acids. In mature green fruits, alanine was most abundant, but glutamic acid, glutamine, serine, leucine, γ-aminobutyric, and other amino acids were present in substantial amounts. Glutamine made up about 40 per cent of the total free amino acid content of young green coconuts. Few differences were found between the free amino acids of fresh and autoclaved coconut water from mature mar-

TABLE II

Nitrogen Content of the Alcohol-Insoluble Residue of Coconut Water

Age of fruits	No. of fruits	Av. quantity of water, ml.	Alcohol-insoluble residue	
			Weight, mg./ml.	Nitrogen content, mg./100 mg.
Young, green	5	400	1.28	0.32
Mature, green	5	253	2.50	0.64
Mature, fresh	37	192	2.50	0.75
Mature, autoclaved	37	192	2.78	1.15

ket fruits, but glutamine was reduced, especially as compared to the content of young green coconuts; γ-aminobutyric acid, alanine, and glutamic acid showed large increases from young to mature green fruits.

The total protein amino acids of coconut water from young green coconuts was 932 μg./100 mg. of alcohol-insoluble residue, 3,762 μg. for mature green coconuts and 4,135 μg. for mature fresh coconuts. Table III indicates the amino acid composition of these proteins. Aspartic acid, lysine, glutamic acid, and arginine are the major amino acids in protein from coconut water of young green fruits; glutamic acid, arginine, and lysine are the main acids in protein from mature green and mature fresh coconuts. In the latter, proline is also present in large amounts, but is found only in trace amounts in mature green fruits. The older fruits (green and fresh) also contained substantial amounts of the leucines, cysteic acid, aspartic acid, glycine, and histidine. In terms of amino acid nitrogen as per cent of total protein nitrogen, coconut water from young green fruits contained more aspartic acid and lysine and less arginine than older coconuts.

TABLE III

PROTEIN AMINO ACIDS OF COCONUT WATER FROM FRUITS OF THREE DIFFERENT AGES

Amino acids	μG./100 mg. alcohol-insoluble residue			Amino acid nitrogen as per cent of total protein nitrogen		
	Young, green	Mature, green	Mature, fresh	Young, green	Mature, green	Mature, fresh
Cysteic	30	279	217	2.47	5.33	3.86
Aspartic	222	205	190	15.89	3.41	2.94
Glutamic	131	797	890	8.49	11.99	12.47
Serine	41	289	166	3.72	6.09	3.25
Glycine	47	183	277	5.96	5.40	7.61
Threonine	18	49	62	1.44	0.91	1.07
Alanine	28	129	61	3.00	3.21	1.41
Histidine	40	163	197	7.37	6.98	7.86
Lysine	142	477	398	18.52	14.44	11.23
Arginine	108	651	663	23.64	33.09	31.40
Proline	13	Trace	478	1.08	Trace	8.57
Valine	18	97	73	1.46	1.83	1.28
Leucines	45	247	246	3.27	4.16	3.86
Phenylalanine	Trace	—	14	Trace	—	0.18
Tyrosine	5	32	92	0.26	0.39	1.05
Hydroxyproline	15	164	Trace	1.08	2.77	Trace
Methionine sulfoxide	29	—	111	2.31	—	1.92
Total	932	3,762	4,135			

The nitrogen content of the alcohol-insoluble residue from various coconut water samples increased with the age of the fruits (Table II). Autoclaving coconut water from mature fresh fruits precipitated additional protein and increased the nitrogen in this sample.

The organic acid composition of coconut water is relatively constant for the three ages of fruits assayed. The data in Table IV show that malic

TABLE IV

NONVOLATILE ORGANIC ACIDS OF COCONUT WATER FROM FRUITS OF DIFFERENT AGES

Organic acids	Meq./ml.			
	Young, green	Mature, green	Mature, fresh	Mature, autoclaved
Shikimic, quinic, and unknowns: A, B	2.51	0.57	0.41	0.30*
Pyrrolidone carboxylic	0.43	0.39	0.18	0.27
Succinic	—	—	0.28	0.18
Malic	9.36	34.31	11.98	14.08
Citric	—	0.37	0.31	0.38
Unknowns	0.27	0.27	0.19	0.16
Total	12.56	35.98	13.33	15.37

* Contains no quinic acid.

acid is the predominant component; it is most abundant in mature green coconuts. Coconut water from young fruits differs from the other samples in having greater amounts of shikimic, quinic, and two unidentified acids; succinic and citric acids were not detected. Quinic acid was found in all samples except the autoclaved coconut water (quinic acid decomposes with boiling). Shikimic and pyrrolidone carboxylic acids were found in all samples.

Data from three other samples of autoclaved coconut water of mature market fruits, taken at different times of the year, confirm the results given in Table IV.

Two unidentified organic acids were found in all samples of coconut water. They were eluted early from the Dowex 1-X10 column, usually with shikimic and quinic acids. The Rf values of these acids were 0.82 and 0.50 in solvent F (isopropyl alcohol—*tertiary* butyl alcohol –benzyl alcohol – water—formic acid, 16:16:48:16:1 v/v) of Stark, Goodban, and Owens (40). The amount of these unidentified acids varied from sample to sample but was most abundant in coconut water from young green fruits.

Approximately one-half the sugar content of coconut water from mature fresh fruits is sucrose; the remainder is fructose and glucose (Table V).

TABLE V

SUGARS OF COCONUT WATER FROM FRUITS OF DIFFERENT AGES

	Mg./ml.			
	Young, green	Mature, green	Mature, fresh	Mature, autoclaved
Sucrose	0.93	9.18	8.90	10.70
Glucose	3.93	7.25	2.46	2.02
Fructose	4.30	5.25	2.51	2.48
Total	9.16	21.68	13.87	16.20

Autoclaving this coconut water did not alter the sugar composition. In the young green coconuts the reducing sugars are abundant as indicated by Child and Nathanael (6), and sucrose is low. More total sugars are found in mature green coconuts than at any other stage of development studied and each sugar is present in substantial amounts. Two high-running spots which reacted as ketoses with naphthoresorcinol-phosphoric acid reagent (4) were found in all samples; these were not identified.

The RNA-P content of coconut water was consistently high in all the samples (Table VI). The amount varied from 20.53 to 35.37 μg./mg. of alcohol-insoluble residue. The DNA-P content was very low in the young coconut water (0.06 μg./mg.) but was consistently higher (2.45 to 3.46 μg./mg.) in coconut water from fruits of other ages.

TABLE VI

RNA-PHOSPHORUS AND DNA-PHOSPHORUS OF THE ALCOHOL-INSOLUBLE RESIDUE FROM COCONUT WATER

Age of coconuts	μG./mg. alcohol-insoluble residue		Ratio RNA-P/DNA-P
	RNA-P	DNA-P	
Young, green	20.05	0.06	342.5
Mature, green	32.82	2.45	13.9
Mature, fresh	35.36	3.46	10.2
Mature, autoclaved	29.77	2.37	12.6

DISCUSSION

Shikimic and quinic acids were detected in samples of coconut water from fruits of three different ages and the greatest amounts were found in young green coconuts. As far as is known, this is the first report of these substances in coconut water. The probable role of these alicyclic acids in aromatic biosynthesis (39, 53) indicates their importance in the developing coconut; it is possible that they also play a role in the nutrition of plant tissue cultures.

Preliminary tests on the growth promoting activity of the organic acids of coconut water have shown some stimulation of tissue cultures of holly stem (*Ilex aquifolium* L.). The growth of holly tissue on media containing organic acids of coconut water from mature market fruits was twice that on the basal medium without the organic acids but only two-thirds that on media containing 10 per cent coconut water. The two unidentified organic acids mentioned previously showed some growth promoting activity, but the results were not conclusive. Hildebrandt, Riker, and Watertor (11) reported a general growth inhibition of tissue cultures on media containing organic acids, but also found that marigold tissue was stimulated.

A substantial amount of sugar is added to tissue culture media when 10 per cent (v/v) coconut water is used (mature fresh coconuts). The total added is about 1.4 grams/l. (890 mg. sucrose, 246 mg. glucose, and 251 mg. fructose). Even greater amounts are present in autoclaved (1.6 g.) and mature green (2.17 g.) coconut water. Besides these energy sources, there are the sugar alcohols, sorbitol, *m*-inositol, and *scyllo*-inositol, isolated by Pollard, Shantz, and Steward (30) and reported to have growth promoting properties.

The high RNA-P content of coconut water relative to the DNA-P content is unusual; the RNA-P/DNA-P ratios are directly related to the high RNA content of coconut water. This would conform to present concepts of the role of RNA in amino acid transport and respiratory metabolism. The RNA of coconut water would carry out these and other functions as part of the metabolic machinery essential to the developing endosperm tissue of the coconut.

Tyrosine was found in the free amino acids of all samples of coconut water; it was also a constituent of all protein samples. Tyrosine was not previously reported for coconut water by Baptist (1), but Shantz (35) isolated it in crystalline form from concentrates of coconut water. Fifteen grams were isolated from 300 gallons of coconut water from green fruits of various ages (about 13.2 mg./l.). Analyses of coconut water shown in Table I indicate that the maximum amount of tyrosine is about 6.4 mg./l. When coconut water is added to media at 10 per cent by volume, the concentration of tyrosine is reduced to 0.64 mg./l. This is low for the nutrition of some tissue cultures as indicated by the finding of LaMotte (14) that the optimal concentration for tobacco callus tissue is 150 mg./l.; this level of tyrosine reversed bud inhibition by indole-3-acetic acid and root inhibition by kinetin.

In addition, Dougall and Shimbayashi (7) have shown a large incorporation of C^{14}-labeled tyrosine by tobacco callus tissue in 24 hours of culturing in a liquid medium. Several protein fractions were found to have different metabolic activities as indicated by the rates of tyrosine replacement. The protein of soluble cell wall material and cell wall residue accumulated more radioactive tyrosine than the soluble protein. These cell wall fractions from callus cultures also contained more nitrogen than similarly treated fractions of nongrowing tobacco pith cells.

It can be postulated, with tyrosine as an example, that certain substances which are essential to controlled differentiation in some plant tissues are not present in adequate amounts in coconut water. Growth may take place, but not cell differentiation. The additional fact that phenylalanine and histidine are present in very small amounts in coconut water indicates another possible limitation for the nutrition of plant tissue cultures. An alternative possibility also exists, i.e., that some amino acids may be present in inhibitory amounts. LaMotte's results (14) suggest that phenylalanine is inhibitory to bud formation in tobacco callus. The low level of phenylalanine in coconut water would, in this instance, be advantageous to tissue proliferation.

The high content of total free amino acids (757.3 μg./ml.) of autoclaved coconut water from mature market coconuts may be significant for the growth of some plant tissue cultures. The level of amino acids in media containing 10 per cent autoclaved coconut water would be about 75.7 mg./l.; using coconut water from young green fruits it would be only 19.0 mg./l. This quantitative difference in amino acids is important, especially when considering the qualitative differences already mentioned (Table I).

Additional information on the role of amino acids in the nutrition of plant tissue cultures is summarized by Gautheret (9). Among more recent work are the results of Sandstedt and Skoog (34) on the effect of the amino acids of yeast extract on tobacco tissue, the arginine requirement of a tissue from the pollen of *Ginkgo biloba* (47), and the tyrosine incorporation studies of Dougall and Shimbayashi (7).

The free amino acids of coconut water were analyzed qualitatively by Baptist (1). His findings are largely confirmed and extended by the quantitative data presented here. He found that alanine was most abundant in young green fruits, whereas glutamine predominated in the samples shown in Table I. This appears to be a matter of the age of the fruits, since the determinations on mature green and market fresh coconuts show that alanine is the dominant amino acid. The data also support the finding (1) that γ-aminobutyric acid is a conspicuous free amino acid in fruits of all ages. Results of analyses of the amino acids from protein of coconut water are in substantial agreement with those reported by Soto Pradera, Fernandez, and Calderin (38). Values for arginine (6.8 μg./mg. alcohol-insoluble residue), tyrosine (0.9), and alanine (0.6) are lower than their findings (12.7, 2.8, and 2.4, respectively); however, acids not detected by them (glycine, methionine, threonine, hydroxyproline, and valine) were found in the present studies. These differences may be due, in part, to the age of fruits analyzed, the method of obtaining protein samples, and analytical procedures. For example, aspartic acid, lysine, and glutamic acid are the major components of protein from coconut water of young green fruits; glutamic acid, arginine, lysine, and proline, from mature market fruits.

The vitamins of coconut water were analyzed by Vandenbelt (49) and the protein amino acids by Soto Pradera, Fernandez, and Calderin (38). Using the results of these analyses, Paris and Duhamet (21) and Paris, Duhamet, and Goris (22) made up media which contained the vitamins and amino acids known to occur in coconut water. Crown gall tissue of salsify grew as well on this medium as it did on media containing whole coconut water (10 per cent by volume). Neither the vitamins nor the amino acids added alone to media gave this response. By contrast, the normal tissues of carrot root gave much less growth on media with the vitamins and amino acids than on media with whole coconut water. This result is not unexpected, however, since crown gall tissues are autotrophic for many substances that are required for normal tissues (Braun, 3).

The growth promoting properties of coconut water are the subject of numerous papers by Steward, Shantz, Pollard, and co-workers (30, 35, 36, 37, 44). Their system of bioassay makes use of explants of the phloem of carrot roots. They have isolated many compounds from a concentrate of 720 gallons of coconut water: 1,3-diphenylurea (37), compounds B, C, D, and E (36), tyrosine, phenylalanine, alanine, leucine, pipecolic acid, and others (35). The 1,3-diphenylurea was a growth promotor in their bioassay, but none of the individual amino acids showed any significant activity. Some of the unknowns were thought to be purines, pyrimidines, or derivatives, and such substances were bioassayed. The results were essentially negative.

Shantz (35) added casein hydrolyzate to media containing coconut water and obtained enhanced growth of carrot tissue, suggesting that perhaps several amino acids in coconut water were present in suboptimal amounts. This is a likely explanation, since Block and Bolling (2) report that the main amino acids of casein hydrolyzate are glutamic acid (21.8 per cent), the leucines (9.7), proline (8.7), valine (7.9), tyrosine (6.6), lysine (6.3), and aspartic acid (6.0). In market fresh coconut water, alanine and γ-aminobutyric acid far exceed glutamic acid and lysine, the next most abundant amino acids.

An increase in the γ-aminobutyric acid in potato tuber tissue in the growing state, as compared to resting tissue, is reported by Steward, Thompson, and Pollard (45). Present results for coconut water and those of Baptist (1) show a similar rise in γ-aminobutyric acid with increasing age of coconut fruits. The results of Steward and co-workers show an increase in hydroxyproline content of protein from proliferating cells as compared to nongrowing tissues. The hydroxyproline content of protein from coconut water from mature green fruits also showed an increase over the content in young green coconuts (Table III). However, a more specific method for hydroxyproline should be used to verify this result.

Growth inhibition by mature fresh or mature autoclaved coconut water has been reported by Norstog (18), Duhamet and Mentzer (8), van Overbeek, Conklin, and Blakeslee (50), and others. Little is known of the substances responsible for this inhibition but embryonic growth in particular seems to be affected. van Overbeek, Conklin, and Blakeslee found three factors of coconut water which affect the growth of *Datura stramonium* embryos: one was thermolabile and caused growth and differentiation; another was heat stabile and caused callus-type growth; the third factor inhibited root growth. The failure of coconut water to promote the growth of potato tuber tissue was overcome by Steward and Caplin (42) through the use of 2,4-dichlorophenoxyacetic acid. More recently, Steward, Mapes, and Mears (43) reported the development of whole carrot plants from freely suspended cells grown in media containing coconut water. Straus and Epp (46), working with a tissue culture of *Cupressus funebris*, found that when gibberellin and acid-hydrolyzed casein were substituted for coconut water in the medium, growth was equal to or exceeded that on the control medium. They suggest that gibberellins or related substances are partly responsible for the growth promoting activity of coconut water.

TABLE VII

Vitamin, Growth Substance, Sugar Alcohol, and Mineral Contents of Coconut Water

Compound	Mg./l.	Reference
Nicotinic acid	0.64	Vandenbelt (49)
Pantothenic acid	0.52	"
Biotin	0.02	"
Riboflavin	0.01	"
Folic acid	0.003	"
Thiamine	Trace	"
Pyridoxine	Trace	"
Auxin	0.07	Paris and Duhamet (21)
Gibberellin	*	Radley and Dear (33)
1,3-Diphenylurea	5.8	Shantz and Steward (37)
Sorbitol	15.0	Pollard, Shantz and Steward (30)
M-inositol	0.01	" " "
Scyllo-inositol	0.05	" " "
	Mg./100 g.	
Potassium	312.0	McCance and Widdowson (15)
Chloride	183.0	" "
Sodium	105.0	" "
Phosphorus	37.0	" "
Magnesium	30.0	" "
Sulfur	24.0	" "
Iron	00.10	" "
Copper	00.04	" "

* A significant growth response was obtained from dwarf peas with an extract of 188 ml. of coconut water.

Some of the constituents of coconut water which are reported in the literature are given in Table VII. These include 1,3-diphenylurea, gibberellin, auxin, vitamins, sorbitol, inositol, and some major and minor elements.

When the amounts of substances given in Table VII are adjusted from mg./l. to about one-tenth this amount, i.e., the concentration which would be present in media containing 10 per cent coconut milk, it is apparent that the auxin and all vitamins except nicotinic acid and pantothenic acid are suboptimal for the growth of most plant tissues. The growth of plant tissue cultures might be improved by the enrichment of some of these substances in coconut water media.

CONCLUSION

The results presented here, as well as those from other laboratories, indicate the known composition of coconut water. There is little doubt that other growth factors, lipid constituents, minor elements, and other components will be found. However, the information already available should assist in the more rational use of coconut water as a supplement in nutrient media. It should also facilitate the design of media of known composition. Media which are better suited to the metabolic requirements of a particular tissue than those containing coconut water are a logical outgrowth of these studies. This is a small but necessary step toward the experimental control of the metabolism, differentiation, and function of plant tissue cultures.

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THE SULFUR METABOLISM OF INSECTS. VI. METABOLISM OF THE SULFUR AMINO ACIDS AND RELATED COMPOUNDS IN THE GERMAN COCKROACH, BLATTELLA GERMANICA (L.)[1]

S. Mark Henry and Richard J. Block[2]

SUMMARY

The metabolism of S^{35}-labeled cystine, cysteine, cysteinesulfinic acid, taurine, methionine, methionine sulfoxide, methionine sulfone, and C^{14}-labeled serine was studied in the German cockroach, *Blattella germanica* (L.). Neither cysteinesulfinic acid nor cysteic acid was used for the synthesis of cysteine. Taurine was partially utilized only after degradation to sulfate by intestinal microorganisms. C^{14}-labeled cysteine was produced by cockroaches injected with serine-C^{14} together with sulfate, sulfite, or thiosulfate. These data indicate that the synthesis of cysteine in the cockroach proceeds via condensation of either sulfide or thiosulfate with serine.

Cysteine was found to undergo the following reactions in the cockroach: incorporation into protein, transsulfuration to methionine when intracellular symbionts are present, and degradation to taurine and sulfate by pathways conforming with those known to occur in other organisms.

Methionine and methionine sulfoxide were found to be interconvertible. Demethiolation, incorporation into protein, conversion to cysteine, and degradation to sulfate and other compounds were observed. Methionine sulfone was not metabolized.

INTRODUCTION

While the house fly, *Musca domestica* L., is known to metabolize sulfur in a manner resembling that of vertebrates (15), the German cockroach, *Blattella germanica* (L.), is different in at least two respects: it can utilize sulfate as the source of sulfur for cystine and methionine (22, 24) and it can convert cysteine to methionine. The intracellular symbionts of *B. germanica* are responsible for sulfate utilization in this insect (21), but the equally surprising conversion of cysteine to methionine is unexplained.

It was the purpose of this investigation to elucidate the mechanisms of synthesis of the sulfur amino acids, their interrelationships, and the role of intracellular and extracellular bacteria in the metabolism of sulfate, cystine, methionine, and related compounds.

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[1] The data presented here represent a part of a dissertation submitted by S. Mark Henry to the Faculty of the Graduate School of Arts and Science of New York University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Aided by grant E-2608, National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland.

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EXPERIMENTAL

INSECTS

Aposymbiotic (symbiont-free) cockroaches were obtained by feeding the insects on a diet containing 0.1 per cent (w/w) aureomycin (8, 21). Xenic cockroaches are those containing both intracellular symbionts and intestinal microorganisms.

RADIOACTIVE COMPOUNDS

Cystine, methionine, and serine. Cystine-S^{35}, methionine-S^{35}, and uniformly labeled serine-C^{14} were purchased from Schwarz BioResearch, Inc., Mount Vernon, N. Y.

Taurine. Taurine-S^{35} was kindly supplied by Dr. P. Fromageot, Saclay, France.

Cysteine. Cysteine-S^{35} was prepared by reducing labeled cystine with an excess of cysteine (14). Any residual cystine was precipitated at neutrality and removed by filtration. This method, however, lowered the specific activity of the compound considerably. In an alternate method, the cystine-S^{35} was dissolved in 0.25N HCl to which was added a few milligrams of zinc dust. After one hour the Zn was removed by filtration. H_2S was bubbled through the filtrate to precipitate the remaining zinc. The small precipitate of zinc sulfide was removed by filtration and the H_2S remaining in solution was displaced with nitrogen. The solution was then dried under vacuum to remove HCl. The crystalline cysteine-S^{35} was dissolved in water just prior to administration to the insects.

Cysteic acid. Cysteic acid-S^{35} was prepared by the oxidation of cystine-S^{35} with peroxide. Five hundred microliters of 30 per cent (v/v) H_2O_2 was added to 1 to 10 mg. of cystine. After 1 hour at 5° C., the sample was dried in an air stream. Water was added and the sample dried again. To assure complete removal of the H_2O_2, 1 ml. of 10 per cent NH_4OH (v/v) was added and the sample was again taken to dryness in a vacuum desiccator. Just prior to injection the cysteic acid was dissolved in water.

Cysteinesulfinic acid. This compound was synthesized from cystine-S^{35} by the method of Lavine (29) scaled down for use with mg. quantities. The cysteinesulfinic acid obtained by this method was about 50 per cent pure as determined by paper partition chromatography. The following method was devised to remove the contaminants, cystine and cysteic acid. A 1 × 30 cm. glass column with a fritted glass disc in the bottom was packed to a height of about 3 cm. with the cation exchange resin, Dowex 50-X8, 200–400 mesh, H^+ form. The resin was thoroughly washed with ethanol and the radioactive sample was applied to the top of the resin in a few hundred microliters of 80 per cent (v/v) ethanol. Absolute ethanol (50 ml.) was passed through the column, collected, and dried. Paper chromatography revealed only cysteic acid in this fraction. Distilled water (50 ml.) was then passed through the column, collected, and dried. This fraction contained about 80 per cent cysteinesulfinic acid and 20 per cent cysteic acid. Cystine remained on the resin.

Methionine sulfoxide. Methionine sulfoxide-S^{35} was prepared by oxidizing methionine-S^{35} with 30 per cent H_2O_2 for sixteen hours at 5° C. The solution was repeatedly dried *in vacuo* to eliminate all of the H_2O_2. The methionine sulfoxide obtained by this method was about 95 per cent pure when assayed by paper chromatography.

Methionine sulfone. Methionine sulfone-S^{35} was made from methionine-S^{35} by performate oxidation according to Toennies (46). A negligible quantity of S^{35}-labeled contaminants was discerned after chromatography of the sulfone in *tertiary* butyl alcohol, methyl ethyl ketone, and water (6, p. 129).

METHODS

The radioactive compounds were administered either by injection or by mixing them with the diet. Eighty per cent (v/v) ethanol extracts and HCl hydrolyzates of the extracted tissues were prepared and chromatographed on paper in 1- and 2-dimensional solvent systems as described previously (21).

In the methionine studies, the formation of mercaptans was determined by connecting an aureomycin-coated flask containing cockroaches injected with methionine-S^{35} to a tube containing 5 ml. of 5 per cent (w/v) cadmium sulfate in N sulfuric acid to trap H_2S. This tube was, in turn, connected to another tube containing 5 ml. of 3 per cent (w/v) mercuric chloride to trap methyl mercaptan (cf. 10, p. 18). The tubes, connected in series, were then subjected to a slight vacuum in order to draw any volatile compounds produced by the cockroach into the mercaptan traps. Collection of the volatile products in this manner was continued for three days at 24° C. At the end of the experimental period, the flask housing the insects was disconnected from the other vessels. Sufficient methyl mercaptan[3] was then bubbled through the $CdSO_4$ trap and into the $HgCl_2$ trap to give a small amount of white precipitate in the latter. Similarly, a small quantity of yellow precipitate was obtained in the $CdSO_4$ trap with H_2S.

The precipitates were collected on filter paper, washed with water and ethanol, and dried with ether. The mercaptides were then quantitatively transferred to planchettes and the radioactivity was determined.

RESULTS

METABOLISM OF CYSTEINE AND CYSTINE

The German cockroach metabolized cystine or cysteine similarly, regardless of the method of administration. The typical radioactivity distribution of cystine metabolites is illustrated in Figure 1. Both cystine and cysteine are converted to glutathione, methionine, taurine, sulfate, and at least one unidentified compound in the xenic cockroach. The distribution in the aposymbiotic cockroach is similar except that neither cysteine-S nor cystine-S is converted to methionine-S.

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[3] CH_3SH was prepared by boiling α-hydroxy γ-methyl mercapto-butyric acid in 70 per cent (v/v) H_2SO_4.

In the experiment from which Figure 1 was prepared, *B. germanica* were fed a diet containing cystine-S^{35} for 1 week. Several insects were sacrificed daily during this period. Inspection of the autoradiograms prepared from chromatograms of the extracts showed a steady disappearance of cystine and an accumulation of glutathione, taurine, and protein-cysteine. Small quantities of methionine-S^{35} were formed also.

METABOLISM OF CYSTEIC ACID

Cysteic acid-S^{35} injected into either xenic or aposymbiotic German cockroaches was largely decarboxylated to taurine. However, a considerable quantity of $S^{35}O_4^{=}$ was present in the fecal material and in the 80 per cent (v/v) ethanol soluble fraction of the insect after injection of cysteic acid-S^{35}. Although taurine-S^{35} was detected less than 24 hours after cysteic acid-S^{35} injection, it was not recovered in the feces until the fourth day. Sulfate, however, was rapidly excreted and easily detected in the first 24 hours. At least one unidentified S^{35}-labeled compound was present in the ethanol extract.

METABOLISM OF TAURINE

The aposymbiotic German cockroach injected with taurine-S^{35} produced sulfate and a small amount of the same unidentified compound formed after injection of cysteic acid. However, xenic *B. germanica* used taurine-S for the synthesis of small amounts of cysteine, glutathione, and perhaps methionine as well as the above compounds. Sulfate was isolated as the benzidine salt (44) after percolating the ethanol extract of the insects through Dowex 50. The benzidine sulfate, thoroughly washed with ethanol, was hydrolyzed with a few drops of 6*N* HCl and the resulting solution was chromatographed. Sulfate was the only S^{35}-labeled substance on the chromatogram.

In an experiment designed to determine the role of intestinal microorganisms in the degradation of taurine to sulfate, 10 aposymbiotic *B. germanica* nymphs were placed on a diet containing 0.1 per cent aureomycin in a beaker, the inside surface of which was coated with aureomycin. After 2 days, 5 of the insects were placed in a clean beaker with normal diet to which were added feces and a macerated intestinal tract of a xenic cockroach. Five days after beginning the aureomycin treatment, the 10 insects and 5 untreated aposymbiotic cockroaches were injected with taurine-S^{35}. The 5 insects still feeding on aureomycin were kept on the

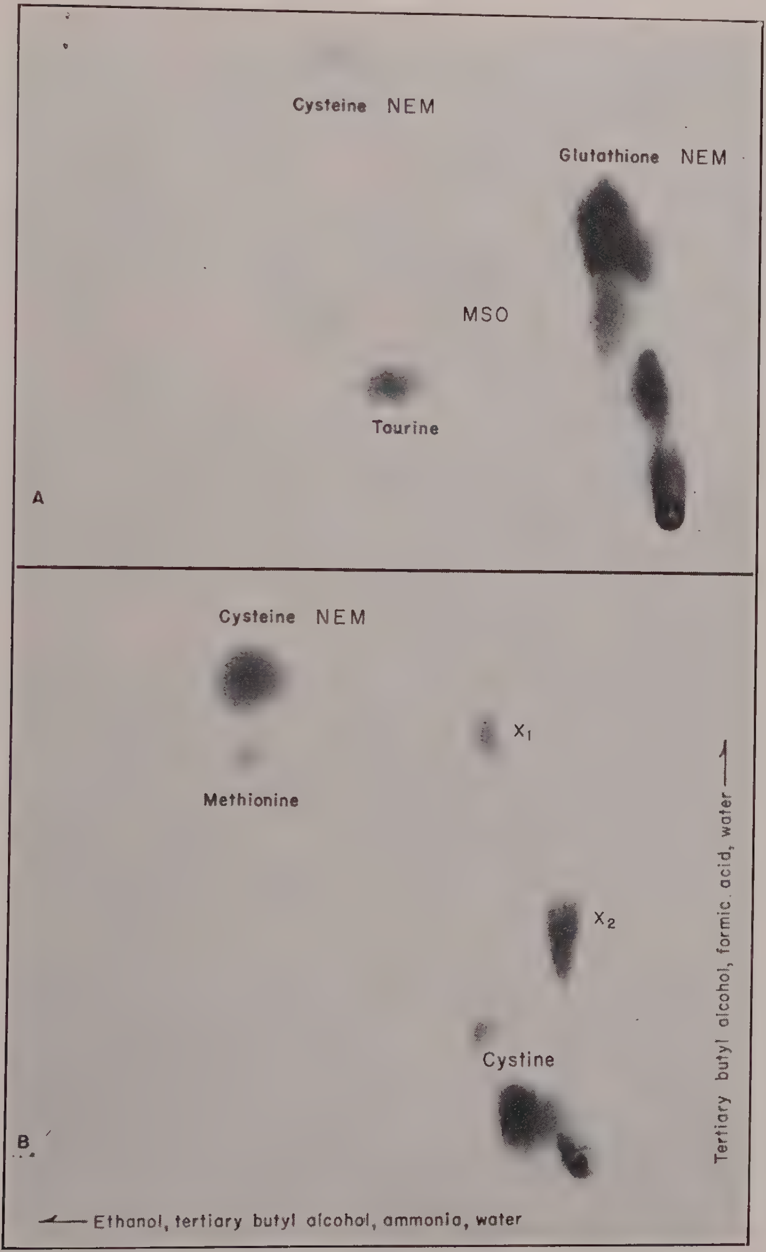

FIGURE 1. Autoradiogram of a chromatogram prepared from xenic *Blattella* fed 1 week on a diet containing cystine-S^{35}. (A) Eighty per cent ethanol extract. (B) Acid hydrolyzate of tissue residue. (Cysteine NEM) cysteine—*N*-ethylmaleimide adduct, (glutathione NEM) glutathione—*N*-ethylmaleimide adduct, (MSO) methionine sulfoxide, (X_1 and X_2) hydrolytic products of cysteine NEM.

TABLE I

EFFECT OF GUT MICROORGANISMS ON DEGRADATION OF TAURINE-S^{35} TO $S^{35}O_4^{=}$ IN APOSYMBIOTIC BLATTELLA

Treatment	Gut microflora population density*	Percentage distribution of S^{35} 3 days after taurine injection			
		In tissue extract		In feces	
		As $S^{35}O_4^{=}$	As taurine	As $S^{35}O_4^{=}$	As taurine
None	Heavy	1	99	9	91
Aureomycin 2 days	Very light	1	99	2	98
Aureomycin 8 days	Very light	2	98	3	97

* Determined by the Gram stain.

antibiotic until the termination of the experiment. After 3 days at 25° C., the 3 groups of insects were homogenized and extracted with 80 per cent ethanol. Feces were also collected and extracted with 80 per cent ethanol. The tissue and feces extracts were percolated through Dowex 50 (H^+) resin and the taurine and sulfate in the anionic fraction were separated by the benzidine method. The relative activities of these fractions were then determined radiometrically (Table I).

In conjunction with this experiment, aposymbiotic *B. germanica*, treated with aureomycin for a 2-day period as described above, were sacrificed on alternate days for a period of 2 weeks and Gram stains of intestinal smears were prepared. The condition, type, and numbers of microorganisms in the gut indicated that most bacteria were destroyed within 48 hours but yeast-like organisms in the gut were not affected. A recovery period of at least 10 days was required in order for residual aureomycin to be eliminated sufficiently to permit reestablishment of the gut flora.

METABOLISM OF CYSTEINESULFINIC ACID

When cysteinesulfinic acid was injected into *B. germanica*, it was almost completely converted to sulfate and taurine in 24 hours. While some of the taurine was undoubtedly formed from the small amount of cysteic acid present as a contaminant in the injected material, the radioactivity of the taurine present 24 hours after injection exceeded that of the cysteic acid in the original solution and must have been formed largely from cysteinesulfinic acid. The position of hypotaurine on the chromatograms of the extracts of both xenic and aposymbiotic insects was determined by cochromatography. Quantitative determination of the radioactivity of the ninhydrin positive hypotaurine spot indicated no S^{35} in this compound in the extract of the xenic insect 24 hours after injection. However, the 24-hour aposymbiotic extract contained a small but significant quantity of labeled hypotaurine.

METABOLISM OF METHIONINE AND ITS OXIDATION PRODUCTS

Methionine. Xenic and aposymbiotic German cockroaches metabolized methionine in a similar manner. Chromatography revealed that most of the compound was oxidized to methionine sulfoxide, but easily detectable quantities of cysteine, glutathione, and sulfate were produced also. Several unidentified compounds were present in both the extract and the feces.

Volatile S^{35}-labeled degradation products of methionine were collected as described in the experimental section. No H_2S^{35} was found but methyl mercaptan was found to be highly radioactive in each of several experiments.

Methionine sulfoxide. Autoradiograms of chromatographed extracts and hydrolyzates of German cockroaches injected with methionine sulfoxide-S^{35} were similar to those obtained from methionine-S^{35} injected insects. Although some of the compound administered was excreted, most of it was converted to methionine and the other metabolites discussed above.

Methionine sulfone. No conversion of methionine sulfone-S^{35} to other radioactive compounds was effected by either the aposymbiotic or the xenic German cockroach. The protein fraction contained no S^{35}.

METABOLISM OF SERINE-C^{14}

Injection of xenic *B. germanica* with uniformly-labeled serine-C^{14} was followed by autoradiographic analysis of extracts and protein hydrolyzates. Within 1 day approximately 25 per cent[4] of the carbon injected was given off as CO_2, and almost 50 per cent was incorporated into protein. The C^{14} distribution was similar in the free amino acids and the protein fraction; serine and glycine were most heavily labeled and smaller amounts of C^{14} were present in glutamic acid, proline and aspartic acid. Weak radioactivity was detected in the tyrosine area. A small amount of labeled cysteine was also found but no cysteic acid.

Total cysteine-C^{14} was determined by adding a few drops of peroxide to a homogenate of *B. germanica* injected 20 hours previously with serine-C^{14}. The homogenate was then hydrolyzed by refluxing in $3N$ HCl for 16 hours and the hydrolyzate was prepared for chromatography by percolating through Dowex 50 (H^+). The anionic fraction was concentrated and chromatographed in a 2-dimensional system using phenol in the first direction and 62 per cent lutidine in the second (41). The radioactivity of the cysteic acid spot was then determined. The data are as follows:

Insect injected with serine alone..................	25	counts/min.
Insect injected with $SO_4^=$ and serine..............	140	"
Insect injected with $S_2O_3^=$ and serine..............	165	"
Insect injected with $SO_3^=$ and serine..............	95	"

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[4] The $C^{14}O_2$, trapped in $1N$ NaOH, was precipitated as $BaC^{14}O_3$.

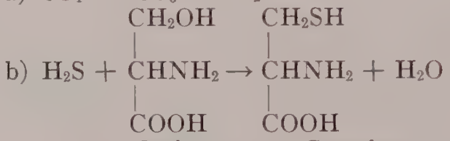
DISCUSSION

SYNTHESIS OF CYSTEINE

Although cysteinesulfinic acid and cysteic acid are known to be formed metabolically from cysteine in many organisms, these compounds are not considered to be important in the synthesis of cysteine. They are certainly not on the main pathway of cysteine synthesis in the cockroach since this insect produced no labeled cysteine from cysteic acid-S^{35} or cysteinesulfinic acid-S^{35}.

Gregory and Robbins (20) state that on the basis of available information a probable scheme for sulfate assimilation as it takes place in yeast and plants is as follows:

a) $SO_4^{=} \rightarrow SO_3^{=} \rightarrow H_2S$

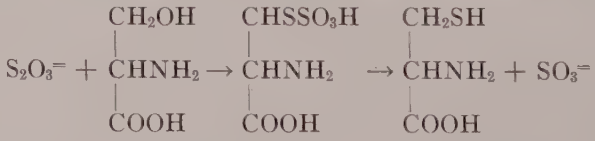


$$\text{b) } H_2S + \begin{array}{l} CH_2OH \\ | \\ CHNH_2 \\ | \\ COOH \end{array} \rightarrow \begin{array}{l} CH_2SH \\ | \\ CHNH_2 \\ | \\ COOH \end{array} + H_2O$$

Serine Cysteine

Reduction of sulfate to sulfite appears to be fairly ubiquitous, occurring in microorganisms (25, 47), in higher plants (18), in birds (12), and in the insect (21). Sulfide formation from sulfate has been demonstrated with cell-free extracts of yeast (25) and bacteria (27, 33). Step b, the incorporation of sulfide into cysteine by way of reaction with serine, has been demonstrated with cell free extracts of yeast by Schlossmann and Lynen (38).

An alternate pathway for the synthesis of cysteine, suggested by Hockenhull (26), involves the reaction of thiosulfate with serine to form cysteine-S-sulfonate which may be hydrolyzed to cysteine and sulfite.



$$S_2O_3^{=} + \begin{array}{l} CH_2OH \\ | \\ CHNH_2 \\ | \\ COOH \end{array} \rightarrow \begin{array}{l} CHSSO_3H \\ | \\ CHNH_2 \\ | \\ COOH \end{array} \rightarrow \begin{array}{l} CH_2SH \\ | \\ CHNH_2 \\ | \\ COOH \end{array} + SO_3^{=}$$

This scheme receives support in the work of Shepherd (40) and Nakamura and Sato (32). While little is known about the mechanisms of biosynthesis of thiosulfate from sulfate there is no doubt that it does actually occur. Neither sulfide-S^{35} nor thiosulfate-S^{35} was detected in cockroaches to which sulfate-S^{35} had been administered. Nevertheless, it may be concluded on the basis of the evidence presented, particularly the C^{14}-serine experiments, that one of the above pathways is operative in these insects.

The small quantity of cysteine-S^{35} formed from injected taurine-S^{35} in these experiments is believed to be a result of degradation of the taurine to sulfite or to sulfate which was then assimilated.

METHIONINE—CYSTEINE—SULFATE INTERRELATIONSHIPS

In order to understand some of the important interrelationships of the various sulfur compounds used in these experiments, a tabular summary of the results is presented (Table II). Some of these data, viz., those pertaining to the synthesis of cysteine, are discussed in the preceding section. The remainder serve as a basis for discussion of methionine synthesis and the oxidative pathways of methionine and cysteine. The results of sulfate injection are taken from a previous report (21).

TABLE II

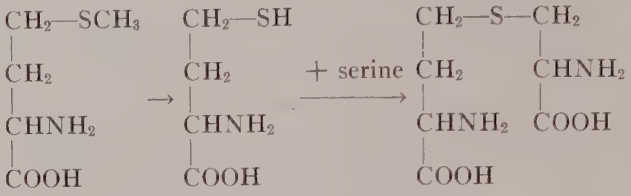
Radioactive Metabolites Observed in the Alcoholic Extracts of Blattella after Injection of Various S^{35}-Labeled Compounds

Compound injected	Physiological condition of insect	Metabolites* into which S^{35} was incorporated					
		Cysteine	Glutathione	Methionine + methionine sulfoxide	Sulfate**	Taurine	Unidentified compounds
Cyst(e)ine	Xenic	+++	+++	+++	+++	++	Present
	Aposymbiotic	+++	+++	–	+++	++	“
Cysteinesulfinic acid	Xenic	–	–	–	++	+	Absent
	Aposymbiotic	–	–	–	++	+	“
Cysteic acid	Xenic	–	–	–	+	+++	Absent
	Aposymbiotic	–	–	–	+	+++	“
Taurine	Xenic	+	++	+	+	+++	Present
	Aposymbiotic	–	–	–	+	+++	“
Sulfate	Xenic	+++	+++	+++	+++	+	Present
	Aposymbiotic	–	–	–	+	–	“
Methionine	Xenic	+	+	+++	+++	–	Present
	Aposymbiotic	+	+	+++	++	–	“
Methionine sulfoxide	Xenic	+	+	+++	+++	–	Present
	Aposymbiotic	+	+	+++	++	–	“
Methionine sulfone	Xenic	–	–	–	–	–	Absent
	Aposymbiotic	–	–	–	–	–	“

* – Indicates absence; +, barely detectable amounts; ++, easily detectable amounts; +++, large amounts as determined by autoradiograms.

** Sulfate is formed from cysteic acid and taurine by the intestinal microorganisms. Details in text.

Methionine metabolism. In addition to incorporation into protein, methionine is known to undergo several other metabolic reactions. Its conversion to cystine has been demonstrated in several organisms including the rat (28, 45). The transsulfuration mechanism involved has been thoroughly studied (cf. 48). It is considered that methionine is first demethylated to homocysteine which then reacts with serine to form a thioether, cystathionine (7).



$$\begin{array}{lclcll} CH_2{-}SCH_3 & & CH_2{-}SH & & CH_2{-}S{-} & CH_2 \\ | & & | & & | & | \\ CH_2 & & CH_2 & + \text{ serine} & CH_2 & CHNH_2 \\ | & \rightarrow & | & \xrightarrow{\qquad} & | & | \\ CHNH_2 & & CHNH_2 & & CHNH_2 & COOH \\ | & & | & & | & \\ COOH & & COOH & & COOH & \end{array}$$

Cystathionine is then split to yield cysteine and an unknown product, probably homoserine. This transsulfuration reaction appears to be virtually irreversible in animals (2, 15).

The present data indicate that the cockroach is like the higher animals in being able to convert methionine to cysteine. Whether the above described transsulfuration process is involved, however, is still a matter of conjecture. The experiments of Hilchey and Cotty[5] show that the xenic cockroach is able to reverse the process and convert cysteine to methionine. The inability of the aposymbiotic cockroach to reverse the process would seem to offer some evidence that the pathway discussed above is operative since only microorganisms are known to be able to convert cysteine to methionine and the pathway is believed to be the reverse of that described for the synthesis of cysteine from methionine (48).

It is probable that in *Aerobacter aerogenes* and *Saccharomyces* there exists a second pathway of methionine synthesis in which the nucleoside, 5′-methylthioadenosine, is an intermediate. This compound appears to be capable of transferring its thiomethyl group to a four carbon compound, such as α-aminobutyric acid, to give methionine. This has been confirmed with the aid of 5′-methylthioadenosine labeled with C^{14} and S^{35} (39) administered to methionine requiring strains of *A. aerogenes*.

Methyl mercaptan (CH_3SH) is known to be formed from methionine (9) but this, of course, cannot explain the presence of S^{35} in the methionine of organisms fed $Na_2S^{35}O_4$. For this to occur, sulfate must be reduced either to cysteine or to methyl mercaptan. Although reduction to cysteine is quite common, methyl mercaptan formation from sulfate is known only in a few organisms such as the wood rotting fungus, *Schizophyllum commune*. The mechanism is believed to be as follows (11):

$$SO_4^{=} \rightarrow SO_3^{=} \rightarrow CH_3SO_3^{-} \rightarrow CH_3SO_2^{-} \rightarrow (CH_3)_2SO_2 \rightarrow (CH_3)_2SO \rightarrow CH_3SH$$

If the symbionts of *B. germanica* are capable of methylating sulfite, methionine might then be produced by the transmethiolating mechanism discussed above. However, since methyl mercaptan has not been detected in cockroaches injected with sulfate and since no organism has been shown to synthesize methionine from sulfate via methyl mercaptan, it is unlikely that this mechanism is operative, at least in insects.

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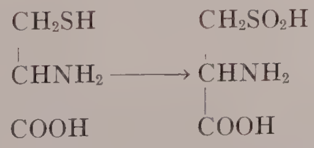
[5] HILCHEY, JOHN D., and VAL F. COTTY, unpublished observations.

Besides incorporation into protein, demethylation, and transmethiolation, methionine in *B. germanica* undergoes oxidation to yield methionine sulfoxide. Chromatography of methionine results in some oxidation to the sulfoxide. The resulting methionine-methionine sulfoxide ratio is similar to that observed on chromatograms of hydrolyzates of cockroach but is much higher than that of 80 per cent ethanol extracts. The large quantities of sulfoxide in the extracts, apparently not due to manipulation of the sample, are surprising inasmuch as there is no proof that enzymes exist which are capable of oxidizing methionine to methionine sulfoxide (48). The function of methionine sulfoxide in *B. germanica* is unknown. However, the compound is known to be an excellent methyl donor (37), and it may very well serve in this capacity in insects.

Reduction of methionine sulfoxide to methionine is a well established fact. Bennett (4) showed that the rat is able to use methionine sulfoxide in place of methionine. The sulfone of methionine is not utilized by higher animals (5) and only one unsubstantiated report of its utilization by yeasts appears in the literature (30). The present results indicate that *B. germanica*, like higher animals, possesses the ability to replace methionine with methionine sulfoxide but not with the sulfone.

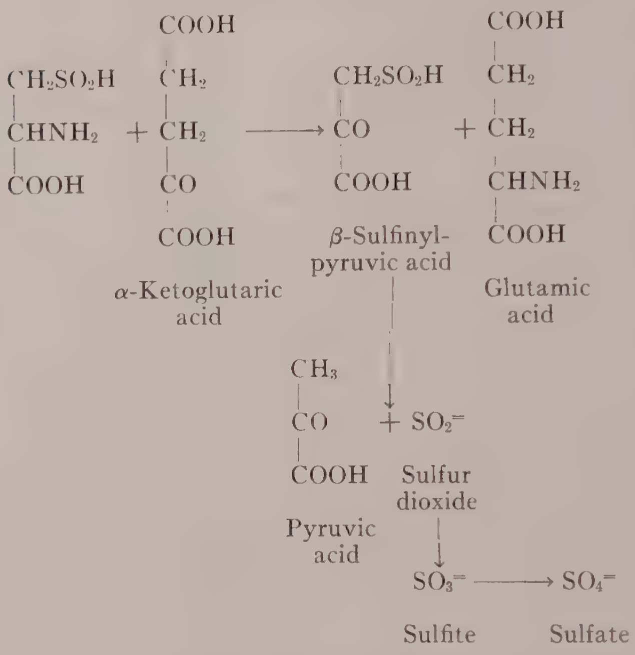
Cysteine metabolism. Cysteine may undergo numerous metabolic reactions. Many of these, such as coenzyme A and mercapturic acid formation and desulfuration, leading to the formation of H_2S and other products (17, 43) have not been demonstrated in insects. Incorporation into proteins and peptides such as glutathione has been shown to occur in *M. domestica* (15) and the experiments herein described show that *B. germanica* uses cysteine or cystine in a similar manner. Utilization of cysteine for methionine synthesis, a characteristic of plants and microorganisms, is discussed above. The remaining important metabolic reactions of cysteine deal with its degradation to taurine, sulfate and other compounds.

The generally accepted pathway for the formation of sulfate from cysteine is outlined below (cf. 48):



$$\begin{array}{c} CH_2SH \\ | \\ CHNH_2 \\ | \\ COOH \end{array} \longrightarrow \begin{array}{c} CH_2SO_2H \\ | \\ CHNH_2 \\ | \\ COOH \end{array}$$

Cysteine Cysteinesulfinic acid

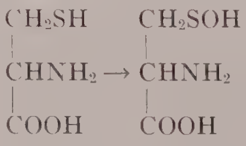


$$\begin{array}{c}CH_2SO_2H\\|\\CHNH_2\\|\\COOH\end{array} + \begin{array}{c}COOH\\|\\CH_2\\|\\CH_2\\|\\CO\\|\\COOH\end{array} \longrightarrow \begin{array}{c}CH_2SO_2H\\|\\CO\\|\\COOH\end{array} + \begin{array}{c}COOH\\|\\CH_2\\|\\CH_2\\|\\CHNH_2\\|\\COOH\end{array}$$

$$\longrightarrow \begin{array}{c}CH_3\\|\\CO\\|\\COOH\end{array} + SO_2^{=} \longrightarrow SO_3^{=} \longrightarrow SO_4^{=}$$

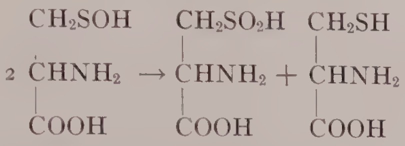
Although cysteinesulfinic acid, the key intermediate in this scheme, has not been detected in *B. germanica*, Table II shows that this compound, when injected into the cockroach, is almost completely degraded to sulfate. Moreover, although some labeled sulfate is found after injection of cysteic acid-S^{35} or taurine-S^{35} the sulfonic acids are excreted largely unchanged. Cysteic acid and taurine may then be ruled out as important precursors of sulfate in this insect.

Cystine is believed to be reduced to cysteine prior to oxidation leading to sulfate (16). The present results indicate that reduction of the disulfide occurs in *B. germanica*. It has been suggested that the formation of cysteinesulfenic acid is the first step in the oxidation of cysteine (31, 34, 35).



$$\begin{array}{c}CH_2SH\\|\\CHNH_2\\|\\COOH\end{array} \rightarrow \begin{array}{c}CH_2SOH\\|\\CHNH_2\\|\\COOH\end{array}$$

The sulfenic acid is unstable and under physiological conditions would be expected to undergo a spontaneous dismutation to cysteinesulfinic acid and cysteine (29, 34, 35).

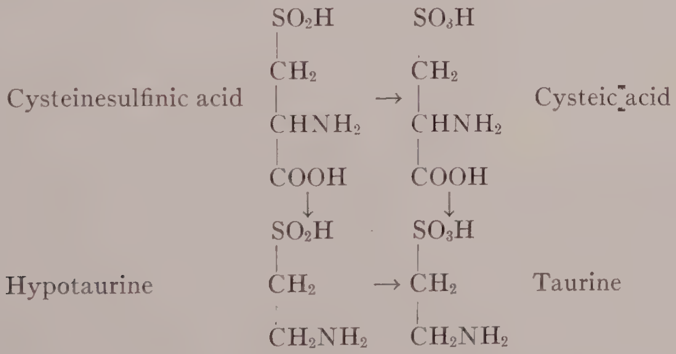


$$2\begin{array}{c}CH_2SOH\\|\\CHNH_2\\|\\COOH\end{array}\rightarrow\begin{array}{c}CH_2SO_2H\\|\\CHNH_2\\|\\COOH\end{array}+\begin{array}{c}CH_2SH\\|\\CHNH_2\\|\\COOH\end{array}$$

Cysteinesulfinic acid is then transaminated to β-sulfinyl pyruvic acid which is enzymatically degraded to sulfate. The results of cystine-S^{35} and cysteinesulfinic acid-S^{35} utilization studies (Table II) offer tentative evidence that this mechanism is operative in the cockroach.

The formation of taurine must also be considered in a discussion of the oxidation products of cysteine. That cysteine may serve as a precursor for taurine has been demonstrated in many organisms including the rat (3) and the house fly (15). Although previous studies (23) failed to demonstrate the ability of the cockroach to degrade cysteine to taurine, the present findings leave no doubt that such ability does exist.

Current concepts of taurine synthesis from cysteine presuppose the formation of cysteinesulfinic acid (42, p. 576). The interrelationships of cysteinesulfinic acid, cysteic acid, hypotaurine, and taurine are outlined below:



$$\begin{array}{lcccl}\text{Cysteinesulfinic acid} & \begin{array}{c}SO_2H\\|\\CH_2\\|\\CHNH_2\\|\\COOH\end{array} & \rightarrow & \begin{array}{c}SO_3H\\|\\CH_2\\|\\CHNH_2\\|\\COOH\end{array} & \text{Cysteic acid}\\ & \downarrow & & \downarrow & \\ \text{Hypotaurine} & \begin{array}{c}SO_2H\\|\\CH_2\\|\\CH_2NH_2\end{array} & \rightarrow & \begin{array}{c}SO_3H\\|\\CH_2\\|\\CH_2NH_2\end{array} & \text{Taurine}\end{array}$$

Decarboxylation of cysteinesulfinic acid to hypotaurine (β-aminoethanesulfinic acid) and then oxidation of the latter appears to be the principal mechanism of taurine formation (3). However, since some organisms, including the rat (3) and the cockroach (Table II), can convert cysteic acid to taurine, an alternate mechanism may exist. Indeed, it has recently been suggested that both mechanisms may be operative in molluscs (1).

In the cockroach, although cysteic acid is converted to taurine, no cysteic acid has ever been detected after administration of either cysteine or cysteinesulfinic acid. Therefore, taurine formation probably occurs in this animal by the usual method of cysteinesulfinic acid decarboxylation and oxidation.

Taurine is generally considered to be an end product of sulfur metabolism. The suggestion that it may serve as an osmoregulator, at least in some organisms, has been made by Allen and Awapara (1). This sulfonic acid may also serve as a sulfur source for some bacteria (36) and yeasts (30). It has also been described as an adequate sulfur source for the xenic cockroach (19). It has apparently been assumed (23, 30) that taurine utilization involves the addition of a carboxy group to form cysteic acid and then reduction of the latter to cysteine. Actually, there is no basis whatsoever for this assumption. Moreover, the data presented here indicate not only that conversion of taurine to cysteic acid does not occur in the cockroach but that, even if it were to occur, the cysteic acid could not be used directly for cysteine synthesis. In addition, it is obvious that taurine is converted to sulfate by microorganisms associated with the cockroach (Table I). When this occurs in the xenic cockroach, the sulfate may then be utilized in the normal manner. Taurine assimilation, therefore, is apparently characteristic only of microorganisms and, until evidence to the contrary is presented, it must be assumed that conversion of the sulfonic acid to sulfate occurs prior to conversion to the sulfur amino acids. The work of Cobey and Handler (13) supports this assumption. Since cysteic acid is converted to taurine any sulfate produced from cysteic acid by the cockroach may also be ascribed to the intestinal microorganisms.

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BIOCHEMICAL STUDIES OF DORMANCY AND AFTER-RIPENING OF SEEDS. II. CHANGES IN OLIGOBASIC ORGANIC ACIDS AND CARBOHYDRATES[1]

Lela V. Barton

SUMMARY

Oligobasic organic acids and carbohydrates were determined in tree peony embryo and endosperm tissue during the breaking of epicotyl dormancy at 5° C. and in controls kept in the greenhouse (ambient temperature 21° to 30° C.) where the epicotyl remains dormant.

Oligobasic organic acids detected were pyrrolidone carboxylic and succinic in small amounts, and malic and citric in much larger quantities. Malic acid accumulated in the endosperm of dormant seedlings to a greater extent than in seedlings after-ripened at 5° C. Embryos held at greenhouse temperature for 4 or 8 weeks also contained more malic acid than those at 5° C. Citric acid content present in dry endosperm tissue increased upon absorption of water by the seed, and then remained fairly constant up to 12 weeks after planting of the germinated seeds. Citric acid content was very low in the embryos of dry seeds, but increased gradually with germination and growth in both the greenhouse and at 5° C. The ratio of citric to malic acid in the endosperm before germination and 12 weeks after planting in the greenhouse was about 1:1. This is in contrast to 3: or 4:1 in embryo tissue.

Sugars detected in the experimental material included fructose, glucose, raffinose, sucrose, and one unidentified sugar. Fructose increased in both endosperm and embryo tissues of seedlings held at 5° C. from 1 to 8 weeks as compared to those in the greenhouse. After 12 weeks, however, fructose content of the endosperm was about the same regardless of temperature, while embryos after-ripened at 5° C. contained at least three times as much fructose as those remaining dormant in the greenhouse. Glucose content of tissues at the two temperatures was the same. Raffinose decreased gradually in endosperm tissue as germination and growth proceeded, and could not be detected in embryos 1 to 12 weeks after planting. The amount of sucrose, the most abundant sugar found, decreased in endosperm tissue upon germination and further growth of the seedling at both temperatures up to 8 weeks after which sucrose continued to decrease in the greenhouse but increased at 5° C. In embryo tissue, on the other hand, there was a marked increase in sucrose from small quantities in the dry or imbibed seeds to a maximum after 8 weeks in the greenhouse and 12 weeks at 5° C. An unidentified sugar in the endosperm and embryos of dry and imbibed seeds persisted in the endosperm tissue during the early stages of germination and growth.

Endosperm tissue held either at 5° C. or in the greenhouse contained large quantities of starch. In embryo tissue, however, starch increased to a greater extent at 5° C. than in the greenhouse.

INTRODUCTION

The dormancy exhibited by certain seeds and their after-ripening during a pretreatment period in a moist medium at low temperature are well-

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known phenomena. However, the biochemical changes which take place during after-ripening have not been elucidated.

Seeds of the tree peony (*Paeonia suffruticosa* Haw.) represent a special kind of dormancy in that they germinate to form roots without any pretreatment, but few green shoots appear above ground unless the germinated seeds are first subjected to a period at low temperature (1). This dormancy of the portion of the seedling which forms the shoot has been called epicotyl dormancy. Morphological changes, i.e. actual growth and differentiation of epicotyl tissues, while exposed to low temperature, cannot account for after-ripening (2), which appreciably depends, rather, upon biochemical changes. An analysis of free amino acid content and its possible significance in the breaking of dormancy has been reported previously (4).

Some of the other biochemical changes which accompany and/or initiate the progressive after-ripening and growth of the dormant epicotyl of tree peony are the subject of the present report.

EXPERIMENTAL PROCEDURES

GROWTH OF TREE PEONY SEEDLINGS FOR ANALYSIS

Nondormant roots were produced by mixing the seeds with moist granulated peat moss and placing them under controlled conditions at a daily alternating temperature of 15° to 30° C. (15° C. for 16 hours and 30° C. for 8 hours daily for 5 days followed by 15° C. for two days each week) which had been shown previously to be satisfactory. Lots of seed were placed under conditions for root production on January 5, 1959. The seeds began to form roots in about 4 weeks. Four lots of seedlings for analysis were removed from the granulated peat moss at intervals of one week beginning on February 13. Seedlings developing four weeks later were not used since it was felt that these may have been less desirable, and a uniform lot of seedlings was essential for the tests. Fresh lots of seeds were placed under the same germination conditions on January 29 and February 26 in order to maintain a good supply of germinated seeds for analysis. Analyses were also made on dry seeds, seeds fully imbibed with water but showing no signs of germination, and germinated seedlings at the stage of planting in soil as described below.

Germinated seeds with healthy roots were removed from the granulated peat moss and were divided into 3 lots with root lengths of 20 to 24 mm., 25 to 28 mm., and 29 to 30 mm. Lots of twenty-five seedlings were planted in soil in 6-inch pots; of these seedlings, twelve to sixteen were 20 to 24 mm. long, six to nine measured 25 to 28 mm., and three to six 29 to 30 mm. Individual measurements were recorded for seedlings of each pot. On each planting date, at least 10 pots were planted; 5 placed in a room at 5° C. and 5 in the greenhouse. At the end of the prescribed period (0, 1, 2, 4, 8, 10, and 12 weeks), two pots each were removed from the cold room and placed alongside two of the same date initially placed in the greenhouse for the purpose of observing green shoot production. The plants in these pots served as physiological controls for the analyses, i.e. a check on the actual progress of the after-ripening process.

The growth of the tree peony seedling below the soil surface after planting and holding in the greenhouse or in a room at 5° C. for one to twelve weeks was measured by determining fresh and dry weights of three lots of twenty embryos each at the end of each of these periods.

PREPARATION OF MATERIAL FOR ANALYSIS

At the end of each test period, the twenty largest of twenty-five seedlings from each of three pots from each temperature were removed from the soil and washed thoroughly with distilled water. Seed coats were removed from the endosperm portion and discarded. The endosperm was then separated from the seedling portion (designated embryo) and fresh weights for twenty endosperm portions and twenty embryos in triplicate were determined.

Each sample was homogenized in a VirTis homogenizer with 25 ml. of 80 per cent ethanol. The homogenate was then centrifuged and washed five times with 80 per cent ethanol. The resulting supernatant fluids were combined.

Free amino acids were isolated on a column of Dowex 50-X8 (4), other organic acids on Dowex 1-X10 (9) while sugars were obtained from the percolate from the two columns. Further treatment to remove fats and other neutral substances from the sugar fraction consisted in drying the fraction, dissolving with small aliquots of water and chloroform, separately and mixed together, centrifuging, and removing the upper water fraction with a pipette. This was followed by washings of the chloroform three times with water, and combining all of the water fractions. These fractions were taken to dryness and stored at −18° C. until they could be made up to suitable volumes and chromatographed for qualitative and quantitative analyses of sugars.

Residues were freeze-dried, weighed and stored in small, tightly-covered vials at −18° C. They were used for determination of starch content to be reported below and will serve for estimation of protein nitrogen at a later date.

ANALYTICAL METHODS

Oligobasic organic acids. Resin columns containing samples of oligobasic organic acids, prepared as described above, were placed on an automatic fraction collector and were eluted using the gradient method (9). The first

100 fractions were obtained by elution with $2.5N$ acetic acid which was followed by elution with $6N$ formic acid to secure an additional 200 fractions. Compressed air was used to give 2-ml. aliquots in 3-minute intervals.

Fractions were held under a stream of air while in a water bath at about 47° C. until dry. The organic acids in each tube were then dissolved in 1 ml. of freshly deionized water, one drop of phenol red was added as an indicator, and the solution was titrated with $0.01N$ NaOH delivered from a microburette. From the data obtained, quantities of the various acids present in the extracts were calculated. Identification of the acids was accomplished by chromatography of the pooled fractions representing each "peak," and comparison with known standards. Tests for isocitric acid showed little if any present in the citric acid fraction.

Sugars. The sugars of each sample were dissolved in 5 ml. of deionized water and were analyzed by placing 20-μl. spots on Schleicher and Schuell No. 598 filter paper and chromatographing by the descending method for 30 hours in the upper phase of *n*-butanol—acetic acid—water (4:1:5 v/v/v). After drying, the chromatograms were cut into strips 1.5-inch wide. Guide strips of known sugars and of the tissue extracts were sprayed with 0.2 per cent resorcinol in 100 per cent ethanol and 85 per cent phosphoric acid (9:1 v/v) mixed just before using; or aniline-phthalate solution (4.98 g. phthalic anhydride; 2.73 ml. aniline; 300 ml. ethanol). Indicative colors were developed in an oven at 93° C. for 2 to 5 minutes for the former reagent and 20 minutes for the latter. Corresponding areas cut from undeveloped strips of the chromatograms were eluted in test tubes with boiling deionized water (5 ml.) and held in a hot water bath (60° C.) for 5 minutes, after which they were filtered through washed glass wool and washed three times with 3 ml. of hot deionized water. The eluates were dried by holding in a water bath at 60° C. under an air stream; the dried sugars were redissolved in 2 ml. deionized water and reacted with 4 ml. of freshly-prepared 0.2 per cent anthrone in concentrated sulfuric acid (15). The tubes were allowed to stand at room temperature for 10 minutes for color development and were then placed in an ice bath to cool before reading the optical density in a Model DU Beckman spectrophotometer at 640 mμ. All readings were made within an hour after placing in the ice bath.

Quantitative values were obtained from standard curves of the sugars concerned. Absorbance readings for 10, 25, 50, 100, 150, and 200 μg. of standards resulted in approximately straight line curves. The best straight line for the points was calculated for each sugar.

Starch. Five mg. each of the freeze-dried residues of the samples were placed in 15-ml. centrifuge tubes. To each tube were added 4 ml. of 52 per cent perchloric acid and 2 ml. deionized water. After 15 minutes the volume was made up to 25 ml. with deionized water and the solution filtered through washed glass wool. Duplicate aliquots of 2 ml. each of the resulting solutions were placed in test tubes for the anthrone reaction as described above for sugars (14). Quantitative values were obtained by comparing absorbance values with those of a glucose standard curve and multiplying by 0.9.

EXPERIMENTAL RESULTS AND DISCUSSION

It should be pointed out that green shoots did not develop in the greenhouse without pretreatment of the germinated seed at 5° C. Furthermore, 1-, 2-, and 4-week periods of 5° C. were not sufficient to after-ripen the epicotyl to permit green shoots to appear. However, 8 and 12 weeks at 5° C. were effective in breaking epicotyl dormancy. This is shown in Figure 1.

The extent of the development of the embryo in soil at 5° C. and in the greenhouse during the first 12 weeks after planting is indicated by the changes in fresh and dry weights. Averages of three replicate determinations are presented in Table I. Weights of corresponding endosperm portions are also given.

It will be noted that there is a progressive increase in the weight of the seedlings with length of time in soil, with a greater increase at 5° C. than at greenhouse temperature. From Table II it is seen that, after 12 weeks in soil, dry weights of roots were about the same whether the seedlings had been held at 5° C. or at greenhouse temperature. The shoot portions (hypocotyl, epicotyl, and cotyledons) held at 5° C., however, were approximately three times as heavy as corresponding shoots in the greenhouse. This was reported earlier (2) at which time morphological studies were made show-

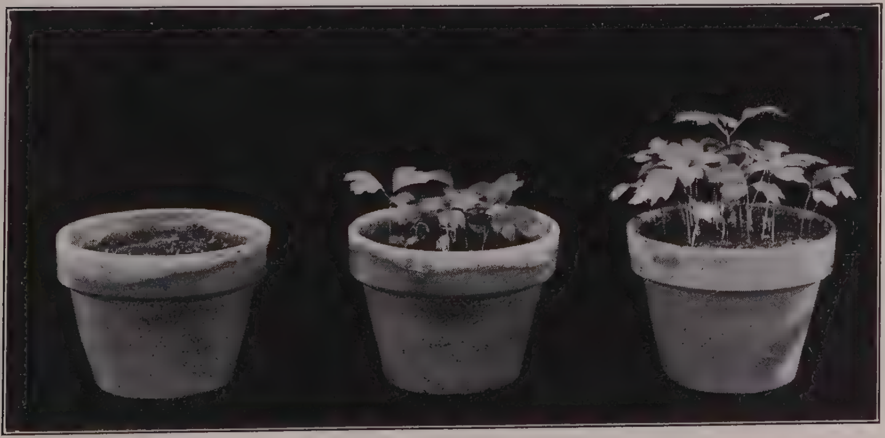

FIGURE 1. Physiological controls for chemical analysis of tree peony seedlings. Seeds which had germinated to form roots were planted in soil and held for (left to right) 4, 8, or 12 weeks at 5° C. before transferring to the greenhouse for green shoot production.

TABLE I

Average Weights of Endosperm and Embryo Portions of Tree Peony Seeds or Seedlings after Various Periods in the Greenhouse or at 5° C. Figures Represent Averages of Triplicate Samples of 20 Seeds Each for All Endosperm and Embryo Portions Except for One Lot of 870 Dry Embryos and One Lot of 500 Imbibed Embryos

Treatment		Average weight in grams of 20 portions*			
		Endosperm		Embryo	
Temp.	Time (wks.)	Fresh	Dry	Fresh	Dry
Dry seeds*		2.85	1.52	0.0124	0.0058
Imbibed seeds*		4.73	1.87	0.0230	0.0038
Germinated seedlings*		4.93	1.78	0.691	0.0306
Greenhouse (21°–30° C.)	1	4.64	1.77	1.02	0.0576
	2	4.43	1.66	1.36	0.0887
	4	4.36	1.61	1.99	0.128
	8	4.43	1.53	3.54	0.249
	12	3.78	1.13	4.71	0.331
5° C.	1	4.73	1.79	0.89	0.0463
	2	4.83	1.76	1.01	0.0582
	4	4.36	1.68	1.43	0.0872
	8	4.12	1.24	4.52	0.351
	12	3.44	0.82	6.42	0.494

* Analyses were made of dry seeds, seeds which had imbibed water but had not germinated, and germinated seedlings as used for planting in soil.

ing a relation between such development and the after-ripening of the dormant epicotyl. The decreasing weight of the endosperm with increased time in soil is to be expected since it indicates the depletion of stored food with development and growth of the embryo.

TABLE II

Weights (g.) of Roots and Shoots of 20 Seedlings of Tree Peony

Treatment		Roots		Shoots		Roots and shoots		Per cent of total wt.			
								Fresh		Dry	
Temp.	Time (wks.)	Fresh	Dry	Fresh	Dry	Fresh	Dry	Roots	Shoots	Roots	Shoots
Germinated seedlings*		0.382	0.0080	0.302	0.0154	0.684	0.0234	55.8	44.2	34.2	65.8
Greenhouse (21°–30° C.)	2	0.817	0.0596	0.537	0.0441	1.35	0.104	60.3	39.7	57.5	42.5
	12	3.06	0.157	1.22	0.123	4.28	0.280	71.5	28.5	56.0	44.0
5° C.	2	0.531	0.0184	0.492	0.0122	1.02	0.031	51.9	48.1	60.1	39.9
	12	3.50	0.186	3.71	0.303	7.21	0.489	48.6	51.4	38.0	62.0

* See footnote in Table I.

OLIGOBASIC ORGANIC ACIDS

For the most part, previous authors have described results of organic acid analyses made on green plants or on seeds during the period of maturation on the plant. Also, usually, entire nondormant seeds, including endosperm and embryo, have been used. In the present tests quantities of oligobasic organic acids were determined for the various fractions of tree peony seeds and seedlings. Calculations were made on the bases of number of seeds, and dry and fresh weights.

Malic and citric acids predominated in all phases of development. Malic acid accounted for 11 to 46 per cent of the total fraction and citric acid from 33 to 86 per cent, depending upon the tissue involved and the time and temperature of treatment. Averages of duplicate determinations, which agreed closely, are given for these acids in Table III. Quantitative measurements were also obtained for pyrrolidone carboxylic acid, 0 to 11 per cent, and succinic acid, 1 to 7 per cent, of the total fraction. Some of the measured pyrrolidone carboxylic acid may have been produced from glutamine when the extract was passed through Dowex 50. A fairly large phosphate fraction (represented by 0.41 to 2.41 ml. 0.1N NaOH required for titration in endosperm tissue and 0.46 to 8.08 ml. in embryo tissue) was not further characterized but showed an increase in all tissues with increased time in soil from 0 to 12 weeks.

Endosperm. Both pyrrolidone carboxylic and succinic acids were present in measurable, though small, amounts in the endosperm at all stages of embryo development. However, the present data warrant no conclusions about the roles of these acids in tree peony epicotyl after-ripening.

In the dry seed malic acid was present in fairly large quantity and increased somewhat upon imbibition of water by the seed (Table III). With germination to form a root, there was a decrease of this acid, further accentuated after 1 or 2 weeks in soil in the greenhouse. After 8 weeks in the greenhouse there was a sharp rise in the amount of malic acid, which continued to a very high level by the end of 12 weeks. In contrast to this, malic acid in endosperm at 5° C. remained more or less constant during the entire 12-week period. Thus, it appears that the accumulation of large amounts of malic acid by endosperm at greenhouse temperatures distinguishes epicotyl-dormant tissue from that after-ripened at 5° C.

Citric acid was present in dry endosperm tissue in somewhat greater quantity than malic acid, but, after an increase in amount upon absorption of water by the seed, no further change was noted up to 12 weeks in the greenhouse. Except for a decrease in citric acid after a week at 5° C., endosperm tissue held at this temperature showed the same general trend as that at greenhouse temperatures.

Embryo. In contrast to endosperm tissue, where some pyrrolidone carboxylic acid was present at all stages, none was detected in embryo tis-

TABLE III

OLIGOBASIC ORGANIC ACID CONTENT OF TREE PEONY SEEDS AND SEEDLINGS AT DIFFERENT STAGES OF DEVELOPMENT. VALUES ARE DUPLICATES OF SAMPLES OF 20 SEEDS OR SEEDLINGS EACH UNLESS OTHERWISE INDICATED

Acid	Treatment		Endosperm			Embryo		
			Mg. acid per			Mg. acid per		
	Temp.	Time (wks.)	20 Seeds	Fresh wt., g.	Dry wt., g.	20 Seeds	Fresh wt., g.	Dry wt., g.
Malic	Dry seeds*		3.11	1.09	2.06	0.04	2.90	6.30
	Imbibed seeds*		4.05	0.85	2.16	0.03	1.32	7.68
	Germinated seedlings*		2.35	0.48	1.32	0.37	0.55	14.15
	Greenhouse (21°–30° C.)	1	1.36	0.31	0.81	0.44	0.42	7.17
		2	1.50	0.35	0.91	0.54	0.36	5.25
		4	2.22	0.49	1.34	1.16	0.57	9.14
		8	3.70	0.80	2.29	2.49	0.70	9.90
		12	5.67	1.48	4.52	2.16	0.49	6.80
	5° C.	1	1.49	0.33	0.26	0.36	0.39	7.31
		2	1.99	0.42	1.15	0.33	0.31	5.25
		4	1.48	0.35	0.90	0.67	0.44	7.29
		8	1.89	0.45	1.47	1.16	0.25	3.19
		12	1.78	0.52	2.18	2.09	0.33	4.37
Citric	Dry seeds*		3.63	1.23	2.36	0.12	9.60	20.50
	Imbibed seeds*		5.06	1.03	2.70	0.24	5.30	31.21
	Germinated seedlings*		3.86	0.81	2.24	0.47	0.69	17.63
	Greenhouse (21°–30° C.)	1	5.10	1.15	3.03	1.30	1.25	21.17
		2	5.60	1.30	3.40	1.50	1.00	14.48
		4	5.30	1.17	3.20	3.40	1.65	23.85
		8	5.45	1.18	3.37	5.51	1.55	22.00
		12	5.90	1.53	5.07	9.00	2.02	28.35
	5° C.	1	3.79	0.84	2.18	1.03	1.10	21.06
		2	5.65	1.18	3.25	1.21	1.15	19.42
		4	4.50	1.05	2.71	3.17	2.10	34.59
		8	5.65	1.33	4.35	5.47	1.19	15.00
		12	5.15	1.51	6.29	6.79	1.08	14.28

* See footnote in Table I.

sue until after eight weeks of growth when measurable amounts were present whether the embryos were grown at 5° C. or at greenhouse temperature.

In the embryo there was an increase in malic acid content from a low in dry or germinating seeds to a much higher value after the seedlings had been in soil in the greenhouse for 4 to 12 weeks (Table III). Malic acid content of seedlings held in soil at 5° C. also increased with time but not as rapidly as in embryos in the greenhouse.

Citric acid content of the dry embryos was very low, but increased with germination and with time in soil in both the greenhouse and the 5° C. room up to a maximum at 12 weeks (Table III).

Roots and shoots of the embryo. Upon removal of the seed coats and endosperm from some of the embryos after 0 (seedling controls), 2, and 12 weeks in soil in the greenhouse or at 5° C., the embryos were further divided into root and shoot portions. The hypocotyledonary portion immediately below the epicotyl, as well as cotyledons, was included in the shoot portion. Data obtained from malic and citric acid determinations on these portions are shown in Table IV.

Shoots of germinated seedlings contained more malic acid than roots, a relationship which also held after 12 weeks in the greenhouse. Twelve weeks at 5° C., however, resulted in more than twice as much malic acid in the roots as in the shoots.

Shoots of germinated seedlings also contained more citric acid than roots; a difference magnified at least threefold after 12 weeks at 5° C. Faludi-Daniel (3) found that malic acid increased in shoots and roots and citric acid in shoots during 2- to 5-day germination of nondormant peas.

From a comparison of the total amounts of malic and citric acids in 20 entire seedlings held in the greenhouse or the 5° C. room (Table III) and the totals of these acids from both roots and shoots (Table IV), it will be seen that these figures determined from replicate seedling lots are in general agreement.

TABLE IV

ORGANIC ACID CONTENTS OF ROOT AND SHOOT PORTIONS OF EMBRYO AT DIFFERENT STAGES OF DEVELOPMENT. SINGLE DETERMINATIONS OF ONE SAMPLE EACH

Acid	Treatment		No. of seeds	Roots			Shoots		
				Mg. acid per			Mg. acid per		
	Temp.	Time (wks.)		20 Seeds	Fresh wt., g.	Dry wt., g.	20 Seeds	Fresh wt., g.	Dry wt., g.
Malic	Germinated seedlings*		50	0.1	0.3	11.5	0.3	0.9	16.9
	Greenhouse (21°–30° C.)	2	40	0.4	0.5	7.2	0.3	0.6	7.7
		12	80	0.7	0.2	4.6	0.9	0.8	7.7
	5° C.	2	40	0.2	0.4	10.4	0.1	0.3	11.5
		12	80	3.1	0.9	16.5	1.2	0.3	3.9
Citric	Germinated seedlings*		50	0.1	0.2	6.9	0.5	1.8	35.1
	Greenhouse (21°–30° C.)	2	40	0.6	0.7	9.5	1.2	2.2	26.5
		12	80	3.6	1.2	23.0	3.6	3.0	29.5
	5° C.	2	40	0.2	0.4	12.8	0.7	1.5	60.1
		12	80	1.4	0.4	7.4	5.6	1.5	18.7

* See footnote in Table I.

Citric acid-malic acid ratios. In the endosperm of dry and imbibed, ungerminated seeds the ratio of citric acid content to malic acid content was about 1:1 (Table III). Upon germination, however, the ratio was 2:1 due to a decrease in malic acid. This general relationship held during growth in the greenhouse up to 8 weeks. Thereafter an increasing quantity of malic acid brought the ratio back gradually to 1:1 after 12 weeks. The amount of citric acid remained constant from the time of imbibition of water by the seed to the end of 12 weeks' growth of the seedling in soil in the greenhouse. With the exception of 1 week, all time periods at 5° C. were also marked by a constancy of citric acid content of the endosperm, but these tissues differed from those in the greenhouse in that malic acid content was at a rather low constant value.

Although the actual quantities of both malic and citric acids were low in the embryos of dry and imbibed seeds before germination, there was a greater proportion of citric acid present. In general, there was three to four times as much citric as malic acid in the embryo throughout its period of growth whether at 5° C. or in the greenhouse. This ratio was maintained by an increase in amounts of both malic and citric acids with time.

The accumulation of both citrate and malate in the embryo with progressive after-ripening may be characteristic of the dormancy-breaking process. Also, the accumulation of malic acid in the endosperm during prolonged dormancy in the greenhouse may be an indication of senescence of the embryo, the epicotyl of which fails to develop at this temperature. A possible relationship is seen here in the progressive increase in malate during senescence of the wheat leaf (12) with no change in citrate during that period. Also malate accumulates most rapidly during the later stages of starvation of rose petals (14). On the other hand, malic acid diminishes rapidly during the first twelve days of the curing period of detached tobacco leaves, while citric acid increases (13). The amounts of malic and citric acids remain constant after twelve days and change little if at all during fermentation and storage of the leaves.

Nikolaeva (8) suggests that the accumulation of organic acids is responsible for the sprouting activity of dormant spindle trees and lentil seeds.

SUGARS

Sugars detected in the experimental material included fructose, glucose, raffinose, sucrose, and one unidentified compound. Identification of sugars was made by comparison with known standards. Fructose and arabinose which migrate to the same Rf in the solvent mixture used were separated by their color response to the resorcinol reagent, since arabinose gave no color with resorcinol spray. Glucose and galactose chromatographed close together but separated enough to be distinct. Variation in the sugar content of the individual seed samples as well as in duplicate determinations of each sample makes the data difficult to interpret. Determination of the standard deviation of the averages of four determinations, consisting of duplicate analyses on each of four extracts expressed as percentage of the average (4, 16), showed that the reliability of the method used for sugar determinations varied with the particular sugar or its quantity while reproducibility of samples depended upon the tissue being analyzed as will be noted below. Statistical comparisons of samples from plants in the greenhouse and at 5° C. were made by a method suggested by Rao (10) using the t test applied to the coefficients obtained by this method. These statistical procedures have allowed the following conclusions to be drawn.

Fructose. The fructose content of the endosperm of the seedlings held at 5° C. for 2, 4, or 8 weeks was shown to be significantly higher than that of those held in the greenhouse for these periods in spite of erratic results. There were no significant differences after 1, 2, or 12 weeks. In the greenhouse series, no fructose was detected after 4 weeks. The peak of fructose accumulation occurred in the endosperm after 4 weeks at 5° C., but there was a decided reduction after 12 weeks.

Although statistical evaluation failed to show fructose content of embryos held in the greenhouse to be different from that of those held at 5° C., there was an indication of a definite increase after 12 weeks at 5° C. over that in the greenhouse. Mori and Nakamura (7) found that the fructose quantity was far greater in potatoes stored at low temperature than in those stored at high temperatures. Significant differences in fructose content occurred at both temperatures depending upon the time of treatment. Embryos of dry or imbibed seeds contained no measurable fructose but with germination small amounts were detected. Upon further growth of the seedlings for one week, this sugar seemed to disappear. Fructose reappeared in embryos after 2 weeks at 5° C. and after 8 weeks in the greenhouse. There was no significant difference in the fructose content of roots and shoots of these embryos.

In the case of fructose determinations of endosperm tissue, it was found that 75 per cent of the total variation was due to the sample and only 25 per cent to the analytical procedure; while error in determining fructose content of embryo tissue was practically reversed, i.e. 35 per cent of the variation was due to the samples and 65 per cent to the procedure.

Glucose. Glucose content of both endosperm and embryo tissues was small, due, probably, to rapid metabolism by the developing plant. Glucose built up in endosperm in the greenhouse from the germinated seedling to 4 weeks after planting after which it decreased at 8 and 12 weeks (Table V). The same general pattern was noted at 5° C.

Except that glucose was still present in the embryos at 5° C. one week

TABLE V

GLUCOSE (μG. IN 0.8 SEED) IN SAMPLES OF TREE PEONY SEEDS AND SEEDLINGS AT VARIOUS STAGES OF AFTER-RIPENING. DUPLICATE DETERMINATIONS OF TWO DIFFERENT SAMPLES ARE GIVEN EXCEPT AS OTHERWISE INDICATED

Treatment		Endosperm				Entire embryo				Roots		Shoots	
		Sample 1		Sample 2		Sample 1		Sample 2		Sample 1		Sample 2	
Temp.	Time (wks.)	a	b	a	b	a	b	a	b	a	b	a	b
Dry seeds*		25	22	18	28	0	0	—	—	—	—	—	—
Imbibed seeds*		35	37	36	28	0	0	—	—	—	—	—	—
Germinated seedlings*		25	17	28	32	21	22	21	34	13	8	8	11
Greenhouse (21°–30° C.)	1	48	48	46	56	0	0	0	0	—	—	—	—
	2	40	48	37	24	0	0	0	0	13	29	20	18
	4	50	56	70	62	24	18	20	24	—	—	—	—
	8	21	41	37	42	33	50	39	40	—	—	—	—
	12	33	29	31	34	27	25	44	28	23	30	19	26
5° C.	1	40	37	33	35	24	23	14	27	—	—	—	—
	2	55	47	34	36	0	0	0	0	18	15	38	31
	4	43	65	40	34	23	17	27	18	—	—	—	—
	8	36	50	42	44	0	25	23	29	—	—	—	—
	12	34	37	38	31	43	59	54	36	35	32	31	44

* See footnote in Table I.

after planting while none was detected in the greenhouse material at this time, there were no significant differences at the two temperatures.

Raffinose. Endosperm tissues in the dry state yielded measurable amounts of raffinose. These amounts more than doubled upon imbibition of the seed but decreased subsequently. At 4 weeks after planting, endosperm tissue held in the greenhouse showed another increase in raffinose content, an increase not evident at 5° C. A decided decrease was noted at both temperatures after 8 and 12 weeks.

Dry and imbibed embryos as well as those of germinated seedlings contained small amounts of raffinose which disappeared completely one week after planting and failed to reappear within 12 weeks in either the greenhouse or at 5° C.

The disappearance of raffinose during the germination of barley (6) and rice (5) has also been noted, and may suggest that this sugar is an important reserve used early in the germination process.

Sucrose. Of all of the sugars detected, sucrose appeared in the greatest quantities in both endosperm and embryo tissue (Table VI). Because of the variation in replicate samples of endosperm tissue, no significant differences in sucrose content in either the greenhouse or 5° C. series could be demonstrated, except that possibly 4 weeks in the greenhouse resulted in higher values than 12 weeks at the same temperature. There appeared to

TABLE VI

SUCROSE (μG. IN 0.8 SEED) IN SAMPLES OF TREE PEONY SEEDS AND SEEDLINGS AT VARIOUS STAGES OF AFTER-RIPENING. DUPLICATE DETERMINATIONS OF TWO DIFFERENT SAMPLES ARE GIVEN EXCEPT AS OTHERWISE INDICATED

Treatment		Endosperm				Entire embryo				Roots		Shoots	
Temp.	Time (wks.)	Sample 1		Sample 2		Sample 1		Sample 2		Sample 1		Sample 2	
		a	b	a	b	a	b	a	b	a	b	a	b
Dry seeds*		660	640	624	648	8	6	—	—	—	—	—	—
Imbibed seeds*		424	444	488	572	2	2	—	—	—	—	—	—
Germinated seedlings*		218	222	208	206	58	59	88	86	12	7	44	58
Greenhouse (21°–30° C.)	1	240	255	235	295	64	50	62	71	—	—	—	—
	2	276	236	175	190	53	48	44	69	38	35	71	64
	4	452	424	304	284	183	158	127	114	—	—	—	—
	8	248	212	160	140	316	292	189	181	—	—	—	—
	12	136	132	148	131	162	165	162	175	60	61	30	34
5° C.	1	345	360	395	415	65	54	60	50	—	—	—	—
	2	405	395	295	270	21	58	35	28	25	24	32	25
	4	315	440	280	356	158	115	151	137	—	—	—	—
	8	184	156	272	284	140	140	74	88	—	—	—	—
	12	598	308	504	510	260	464	255	310	79	72	24	30

* See footnote in Table I.

be a decrease upon germination and further growth at both temperatures, especially when compared to the quantity in endosperm of dry seeds. This is to be expected since sucrose, also, is usually considered as one of the first substrates to be oxidized upon initiation of the processes leading to germination.

In embryo tissue, on the other hand, there was a marked increase in sucrose from small quantities in the dry and imbibed seeds to an apparent maximum after 8 weeks in the greenhouse and 12 weeks at 5° C.

Sucrose in the root portions of the embryo increased at least 5-fold during the 12-week period after the seedlings were planted in soil, regardless of the temperature (Table VI). The shoot portions, on the other hand, contained much larger amounts than the roots at the time of planting (germinated seedlings). This amount increased still further in shoots held in the greenhouse for two weeks after which a decrease was noted.

Unidentified compound. Fairly large quantities of an unidentified sugar were detected on the chromatograms at an Rf value (about 0.10) below that of raffinose in certain of the samples. This sugar gave a color test with the resorcinol but not with the aniline-phthalate reagent. After hydrolysis with invertase, only fructose was detected.

The unidentified compound was found in the endosperm of dry, imbibed, and seedling controls and of seedlings after 1, 2, or 4 weeks in either

TABLE VII

STARCH CONTENT OF SAMPLES OF TREE PEONY SEEDS AND SEEDLINGS AT VARIOUS STAGES OF AFTER-RIPENING. DETERMINATIONS ON DUPLICATE LOTS OF SEEDS

Treatment		μG./20 seeds						μG./mg. dry weight					
Temp.	Time (wks.)	Endosperm		Entire embryo		Roots	Shoots	Endosperm		Entire embryo		Roots	Shoots
		a	b	a	b			a	b	a	b		
Dry seeds*		230	139	0.4	—	—	—	138	115	73	—	—	—
Imbibed seeds*		232	203	0.4	—	—	—	135	98	103	—	—	—
Germinated seedlings*		326	274	10	7	2	3	178	160	328	305	265	213
Greenhouse (21°–30° C.)	1	294	313	20	20	—	—	175	168	330	298	—	—
	2	292	274	26	29	21	14	163	180	243	293	345	328
	4	369	286	43	26	—	—	21·5	178	332	206	—	—
	8	287	326	65	70	—	—	178	200	269	269	—	—
	12	236	188	64	69	30	28	180	183	219	206	194	231
5° C.	1	305	386	13	15	—	—	175	208	273	300	—	—
	2	329	291	19	18	6	4	188	170	305	288	310	298
	4	271	291	20	23	—	—	165	175	263	256		—
	8	220	211	134	135	—	—	153	180	369	369	—	—
	12	133	137	138	166	48	55	157	160	300	313	256	181

* See footnote in Table I.

greenhouse or 5° C. It was found in largest amounts in the dry and imbibed seeds and was reduced thereafter. It was not detected in endosperm of seedlings 8 or 12 weeks after planting.

In the embryo, this compound was present in dry seeds but there was only a trace in imbibed seeds and none after germination.

STARCH

Starch content of the samples is shown in Table VII. Endosperm tissue contained large quantities of this compound with no apparent effect of temperature on the quantities present.

In embryo tissue, however, starch increased from a small quantity in dry or imbibed seeds to a maximum 8 weeks after planting the seedlings. The amount present in seedlings at 5° C. after 8 or 12 weeks was approximately double that in the greenhouse. This may represent a greater conversion of sucrose to starch at the low temperature. It has been noted by Rumi and Rivoir (11) that wheat is capable of greater carbohydrate synthesis after low temperature treatment (vernalization).

Although certain differences in the chemical composition of endosperm and embryo tissues from dormant and after-ripened tree peony seeds have been found, it is not known at the present time whether these are directly related to the dormancy-breaking process. Further analyses of nitrogen metabolism and growth regulators are now being made in an attempt to elucidate this phenomenon.

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COLORIMETRIC DETERMINATION OF MICRO AMOUNTS OF DIMETHYL TETRACHLOROTEREPHTHALATE

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W. E. Priddle[2], and Sally Klein[3]

SUMMARY

A procedure is described for the photometric analysis of the pre-emergence herbicide dimethyl tetrachloroterephthalate by causing it to react with γ-(4-nitrobenzyl)pyridine in the presence of pH 4 phthalate buffer for one hour at 120° C. followed by addition of pyridine and triethylamine. Concentrations from 1 to 4 μg. per ml. gave colors which followed Beers' law satisfactorily and had an absorption maximum at 565 mμ. Development of optimum reaction conditions is described. Dimethyl tetrachloroterephthalate can be extracted from soil and plant homogenates by dichloromethane. Most interferences can be removed by successively partitioning between acetonitrile and *n*-heptane, decolorizing with Norit A and chromatographing on activated alumina. Recoveries of 75 to 100 per cent were obtained from soil and plant homogenates.

INTRODUCTION

Several years of field trials have demonstrated the utility of dimethyl tetrachloroterephthalate as a pre-emergence herbicide for use on crops (5, 6). This fact necessitated the development of a reliable analytical procedure which could be used to detect dimethyl tetrachloroterephthalate independently of possible degradation products and other pesticides. Since dimethyl tetrachloroterephthalate is the methyl ester of a strong organic acid, it was felt that it might be a powerful enough alkylating agent to react with γ-(4-nitrobenzyl)pyridine (4-NBP) and thereby yield a colored product similar to those previously used for the detection of ethylenimines and alkylating agents (3). It has been shown by Koenigs, Köhler, and Blindow (4) that γ-(4-nitrobenzyl)pyridine reacts with methyl iodide to form a quaternary salt which yields a blue dye in alkaline solutions. Using this information, it was possible to obtain a color reaction suitable for quantitative analysis of dimethyl tetrachloroterephthalate by modifications of the reaction conditions.

Extraction and purification procedures have been developed for isolating the herbicide from plant homogenates and from soil for colorimetric analysis.

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MATERIALS AND METHODS

Chemicals. Pure samples of dimethyl tetrachloroterephthalate were obtained from Diamond Alkali Company. The preparation of this chemical and other related compounds will be described elsewhere.

The following solvents and reagents were used: Reagent grade pyridine (Mallinckrodt Chemical Works), reagent grade triethylamine (Eastman), Clark and Lubs pH 4 phthalate buffer (2, p. 200) (Beckman Instrument Company), (A. R.) acetone (Mallinckrodt Chemical Works), reagent grade methylene chloride (several suppliers), neutral Norit A decolorizing carbon (Fisher Scientific Company), commercial grade acetonitrile, reagent grade *n*-heptane (several suppliers), anhydrous sodium sulfate (Fisher Scientific Company), and grade 1 Woelm acid aluminum oxide (Alupharm Chemicals, New Orleans, La.).

The 4-NBP was prepared by Dr. Edward A. Prill at Boyce Thompson Institute, but it can also be obtained from Mathieson Coleman and Bell. The reagents used in the analytical determinations were: 5 per cent solutions of 4-NBP in (A. R.) acetone (w/v) and triethylamine reagent [50 per cent solution of triethylamine in pyridine (v/v)].

Apparatus. A Bausch & Lomb Spectronic "20" direct reading colorimeter was used in making most of the measurements in the development of the method. Absorbances also were measured with a Beckman Model B spectrophotometer with matched 1-cm. Corex cells. An autoclave operated at 120° C. at 18 pounds pressure was used for carrying out the reaction. However, a home pressure cooker will also serve satisfactorily. Special laboratory glassware which is desirable includes 1,000-ml. and 250-ml. separatory funnels, 4-inch funnels, fritted funnels medium porosity Büchner type, filter flasks with side tube, quart jars with lead or aluminum lined lids, and 22 mm. o.d. × 28 cm. glass columns with stopcocks. Since the alkylation reaction is carried out in 125-ml. Erlenmeyer flasks, a large supply of these is desirable. Other apparatus in addition to common laboratory glassware includes an interval timer, source of clean dry air, aluminum foil, fluted Whatman No. 802 filter paper, hot plate for water bath or suitable substitute, and a one-gallon Waring Blendor Model CB-4.

Colorimetric analysis. Prepare a stock solution of pure dimethyl tetrachloroterephthalate in acetone at a concentration of 1,000 μg. per ml. From the stock solution prepare solutions containing 10, 20, 30, and 40 μg. per ml. of acetone. Add one ml. of each solution to duplicate 125-ml. Erlenmeyer flasks. Evaporate to dryness with clean, dry air. To each residue add 2 ml. of 5 per cent 4-NBP reagent plus 1 ml. of Beckman pH 4 phthalate buffer diluted to half strength. Cover the flasks with aluminum foil and place them in an autoclave at 120° C. for one hour. After the flasks have been removed from the autoclave and cooled, remove the aluminum covers and evaporate the contents to dryness with a stream of clean, dry air. Add 1 ml. of the pH 4 buffer and 8 ml. of pyridine to each of the dried residues. Swirl until all of the solid dissolves and then add 1 ml. of triethylamine reagent. Mix thoroughly and determine the absorbance at 565 mμ immediately against a reagent blank which is processed in the same manner. A standard curve is obtained by plotting absorbance against μg. of dimethyl tetrachloroterephthalate per ml. of the final solution.

Extraction from plant material and soil. The purpose is to give a preliminary but workable general extraction and purification procedure which has been satisfactory in obtaining recoveries when known amounts of chemical were added to soil and plant homogenates. Extract 200 grams of the prepared sample with 500 ml. of methylene chloride. For soft succulent products such as vegetables and berries, add the sample to a Waring Blendor and macerate for 2 minutes. Without stopping the blender, rapidly add 500 ml. of methylene chloride and continue blending for 3 minutes. Transfer the homogenate to a 1,000-ml. separatory funnel. Allow the phases to separate and draw off the solvent layer and save for purification.

To prepare hard fibrous tissues for extraction put them through a food chopper. For such material and soil, place 200 grams in a 500-ml. glass-stoppered Erlenmeyer flask, add 250 ml. of methylene chloride and shake for 2 hours on a mechanical shaker. Remove from the shaker and filter the solvent through four thicknesses of cheesecloth. Reserve the filtrate for the purification procedure.

Purification procedure. Transfer 250-ml. aliquots of the methylene chloride extract to a 400-ml. beaker and evaporate to dryness using a current of clean, dry air. Add 50 ml. of acetonitrile and mix well with a stirring rod. Transfer the solvent to a 250-ml. separatory funnel. Wash the residue with 25 ml. of *n*-heptane and add to the acetonitrile in the separatory funnel. Stopper and shake for 1 minute allowing the phases to separate. Transfer the lower phase (acetonitrile) to a second separatory funnel and extract with 25 ml. of *n*-heptane for 1 minute. Repeat the procedure until three extractions with *n*-heptane have been made.

Transfer the acetonitrile phase to a 250-ml. beaker and evaporate to dryness under a stream of clean, dry air. A hot water bath (50° C.) is used to facilitate the evaporation of the solvent. Dissolve the residue in 50 ml. of methylene chloride and add 2 tablespoons of decolorizing charcoal (Norit A). Mix thoroughly and allow the solution to remain in contact with the adsorbant for 15 minutes with occasional stirring. Filter with the aid of a vacuum through fritted disc funnels (medium porosity) into a 250-ml. suction flask. Wash the beaker and funnel with two 15-ml. portions of methylene chloride. Transfer the filtrate to a 250-ml. beaker and rinse the suction flask with 20 ml. of methylene chloride and add to the filtrate in the beaker. The filtrate (100 ml.) is reserved for passage through the chromatographic column.

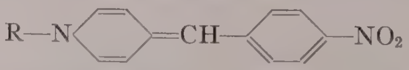
Preparation of chromatographic column. Place a plug of glass wool in the bottom of the column and force into place with a solid glass rod. Pack the column with activated alumina to a height of 4 inches. During the packing, compact each inch of material as it is treated. On top of the 4 inches of activated alumina, place 1 inch of anhydrous sodium sulfate. Precondition the column by passing through 150 ml. of methylene chloride. Do not allow the level of the methylene chloride to drop below the top of the sodium sulfate layer. The columns can be conditioned in advance, but should be used the same day.

Percolate the decolorized methylene chloride containing the residue through the column. The dimethyl tetrachloroterephthalate is adsorbed on the activated alumina. After all of the methylene chloride has been transferred to the column, add 25 ml. of 2 per cent acetone in methylene chloride (v/v) to the beaker which had contained the methylene chloride and transfer it to the column. As soon as the meniscus of the methylene chloride reaches the sodium sulfate layer, wash down the sides of the column with 2 to 3 ml. of the 2 per cent acetone solution. Repeat this procedure three times and allow each washing to pass into the sodium sulfate layer before adding the next. A fourth 25-ml. washing can be added to the column directly. Discard the solvent which has passed through the column up to this point. When the last 25-ml. rinse reaches the sodium sulfate layer, add an additional 100 ml. of 2 per cent acetone solution to the column and collect the eluate. The dimethyl tetrachloroterephthalate will be eluted from this column in the last 100 ml. of 2 per cent acetone solution.

When the last of the solvent has passed into the sodium sulfate layer, remove the collection flasks. Add 2 ml. of 4-NBP solution to the eluate and evaporate the solution to dryness in a 50° C. hot water bath under a stream of clean, dry air. Add 1 ml. of buffer, cover with aluminum foil, and heat for 1 hour at 120° C. (15 to 18 pounds pressure) in an autoclave or other suitable sterilizer. Develop and measure the color in the same manner as described for the standards.

RESULTS AND DISCUSSION

Development of method. Several alkylating agents have been shown to form dye precursors when reacted with 4-NBP as described by Epstein *et al.* (3) and reviewed by Burchfield and Schuldt (1). The chemical identity of the dye has been determined by Holzman.[4] Epstein *et al.* (3) isolated the colored materials formed in the reactions between butyl, heptyl, and decyl bromides with 4-NBP. The dye was assigned the following structure:



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[4] HOLZMAN, G. Office of Scientific Research and Development, OSRD 4288, October 27, 1944 (unpublished data).

The color is developed through dehydration rather than ring cleavage as in the pyridine-alkali reaction. Impetus for trying 4-NBP as a reagent for detecting dimethyl tetrachloroterephthalate came as a result of failure with the pyridine-alkali method when a number of modifications were tried. It was felt that dimethyl tetrachloroterephthalate might function as a methylating agent rather than through active halogens because of the strong acidity of tetrachloroterephthalic acid.

When dimethyl tetrachloroterephthalate was treated with a 5 per cent solution of 4-NBP in acetone in a boiling water bath for periods up to two hours, extremely variable results were obtained. It appeared that color could be developed more consistently after heating when the residue was taken up in pyridine rather than acetone as suggested by previous workers. Color was obtained with one preparation of 4-NBP, but not with another.

TABLE I

EFFECT OF pH VALUE OF BUFFER ON COLOR INTENSITY

pH of buffer	Absorbance at various concns. of dimethyl tetrachloroterephthalate (μg./ml.)			
	0	1	2	3
1	0.020	0.075	0.150	0.235
2	0.025	0.105	0.205	0.340
3	0.035	0.125	0.225	0.315
4	0.060	0.155	0.250	0.380
7	0.035	0.085	0.195	0.205

In cases where color was obtained, increasing the length of the heating period enhanced color intensity, but sensitivity was not satisfactory. The addition of Beckman phthalate buffer pH 4 to the reactants before heating in the water bath resulted in reproducible results, but the sensitivity was still poor. When buffers with higher or lower pH values were tried, color intensity decreased (Table I). It was apparent that color development was dependent upon the acidity of the medium. Evidently impurities in the 4-NBP caused the variable results obtained in earlier experiments since the highly purified reagent did not yield strong colors in the absence of buffer. Phthalate buffers were superior to phosphate buffers for this purpose. Beckman pH 4 buffer gave optimum color intensity. When this buffer was used along with a 5 per cent solution of 4-NBP in pyridine, color intensity increased with time in the boiling water bath. This suggested the use of higher temperatures. Therefore, samples were placed in an autoclave for 30 minutes at a temperature of 120° C. (18 p.s.i.). Under these conditions, color intensity increased 5-fold.

Experiments were then conducted to determine the time required to obtain maximum color development. It was found that 60 minutes in an

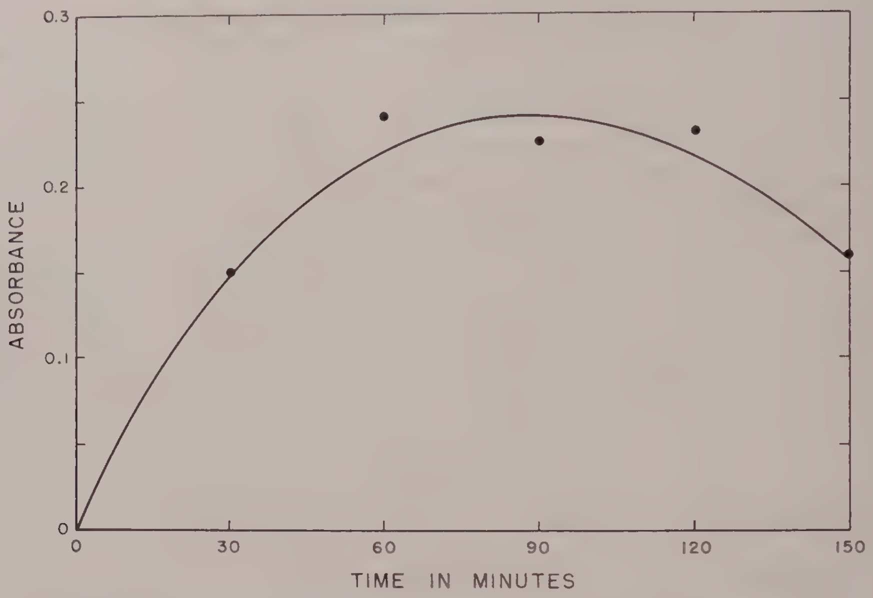


FIGURE 1. Effect of heating time in autoclave on color intensity at 2 μg./ml. of dimethyl tetrachloroterephthalate read at 565 mμ.

autoclave at 120° C. is optimum (Fig. 1). The effect of buffer pH on color intensity was then rechecked, and it was found that color intensity at 565 mμ is virtually independent of pH between values of 3 and 5. Similarly, dilution of the buffer to one-fifth strength did not diminish absorbance. Therefore, Beckman pH 4 phthalate buffer at half strength was used in all subsequent work. Varying the concentration of the 4-NBP reagent between 3 and 10 per cent also failed to produce significant changes in intensity (Table II). Consequently a 5 per cent solution of 4-NBP in acetone was selected arbitrarily. This is the same concentration used by Epstein

TABLE II

EFFECT OF CONCENTRATION OF γ-(4-NITROBENZYL)PYRIDINE REAGENT IN ACETONE (W/V) ON INTENSITY OF COLOR PRODUCED

Per cent reagent	Absorbance	
	2 μG./ml.	4 μG./ml.
0.1	0.068	0.097
0.5	—	0.521
1.0	0.244	0.560
3.0	0.262	0.593
4.0	0.259	0.605
5.0	0.270	0.610
6.0	0.264	0.584
10.0	0.271	0.545

et al. (3) in their work with ethylenimines and other alkylating agents.

The stability of the reaction product formed on heating dimethyl tetrachloroterephthalate with 4-NBP and buffer was then investigated. Pyridine and triethylamine were added to a sample immediately after autoclaving and to a second sample held at room temperature 5 hours after autoclaving. No significant difference in color intensity was observed. Therefore, it is permissible to allow samples to stand in the dry state at least 5 hours before developing color. The reaction product was then dissolved in pyridine and triethylamine added immediately and after an interval of 1 hour. Again no significant differences in color intensity were noted, indicating that the length of time between adding the reagents is not critical. Some color is developed when the reaction product is dissolved in pyridine, but absorbances are not reproducible. Color intensity and reproducibility are enhanced greatly on addition of triethylamine. Readings should be made shortly after adding this reagent since the color fades on standing (Table III).

TABLE III

STABILITY OF COLOR AFTER ADDITION OF 1 ML. TRIETHYLAMINE-PYRIDINE (50 PER CENT V/V) TO RESIDUE TAKEN UP IN 10 ML. OF PYRIDINE AT A CONCENTRATION OF 2 μG./ML. OF DIMETHYL TETRACHLOROTEREPHTHALATE

Time in minutes after addition of triethylamine	Absorbance at 565 mμ
0	0.270
2	0.203
5	0.184
7	0.165
10	0.146

Samples containing as much as 1,000 μg. of dimethyl tetrachloroterephthalate can be diluted to concentrations suitable for colorimetric measurements (2 to 4 μg./ml.) without loss of accuracy. However, care must be taken that all of the reaction product is brought into solution. This is accomplished by heating the residue with pyridine in a boiling water bath for a few minutes. It was shown that this treatment has no effect on color intensity. The sample can then be diluted to the required concentration with pyridine. The need for dilution will be apparent if a strong blue color develops on adding pyridine (8 ml.) in the absence of triethylamine. When this occurs, the volume should be brought up to 9 ml. with pyridine, and an aliquot of this solution diluted to a suitable concentration with pyridine. Color is then developed by adding triethylamine in the usual way.

After autoclaving, it is advisable to dry the residue under a current of clean, dry air and add exactly 1 ml. of pH 4 buffer to insure that a constant volume of water is present. The water must be present to avoid turbidity arising from the buffer salts. Acetone cannot be used in place of pyridine as a medium for developing color. The reaction product formed on the addition of triethylamine has an absorption maximum at 565 mμ (Fig. 2).

Beers' law is followed accurately at concentrations of 1 to 4 μg. per ml. (Fig. 3). However, day-to-day variations in specific absorbance as measured over a 3-month period were significant at the 1 per cent level so it is necessary to run a standard curve with each set of unknowns for optimum results. In-day variation was not significant at the 1 per cent level, indicating excellent agreement between duplicate samples on any given day.

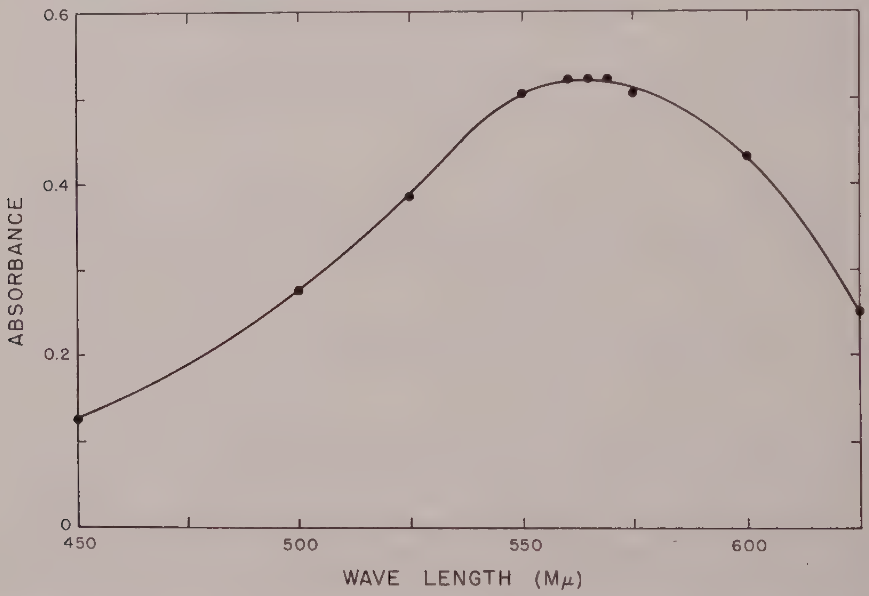


FIGURE 2. Absorption spectrum of reaction product of dimethyl tetrachloroterephthalate with γ-(4-nitrobenzyl)pyridine and triethylamine in pyridine solution.

Other derivatives of terephthalic acid give colored products under these conditions, but in general the response is not as intense as with dimethyl tetrachloroterephthalate (Table IV). Color decreases when fewer chlorine atoms are substituted in the aromatic ring or when the methyl groups of the ester are replaced by higher alkyl groups. In general, modifications that would be expected to reduce the alkylating power of the terephthalate ester result in lower intensities. Compounds which respond sufficiently to the color test to be classed as potential interferences include dimethyl trichloroterephthalate, methyl butyl tetrachloroterephthalate,

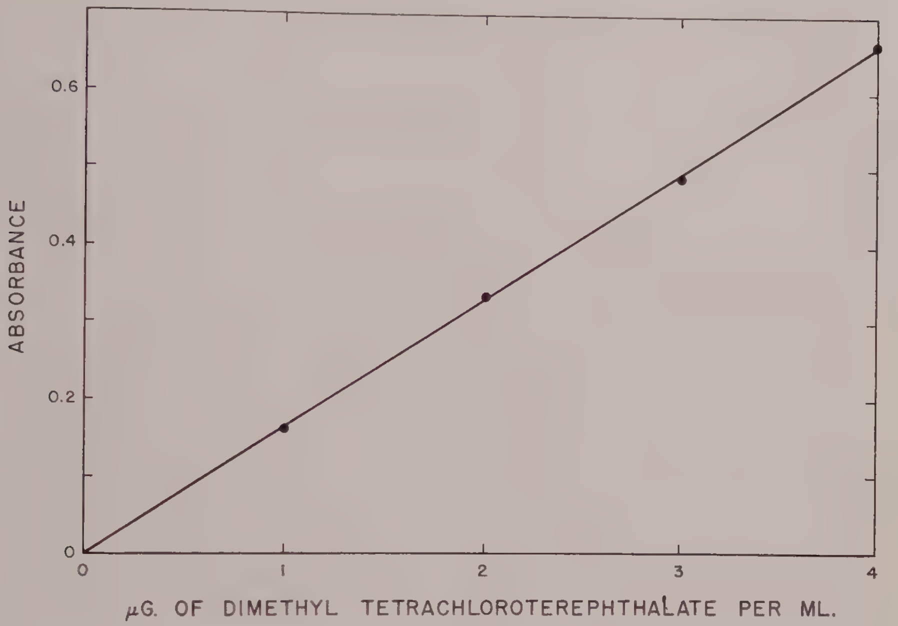
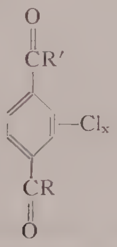


FIGURE 3. Standard curve for the estimation of dimethyl tetrachloroterephthalate.

TABLE IV

SENSITIVITY OF VARIOUS TEREPHTHALIC ACID DERIVATIVES TO THE γ-(4-NITROBENZYL)PYRIDINE METHOD



R	R′	x = No. of chlorine atoms	Relative color intensity
OCH_3	OCH_3	4	10.0
OCH_3	OCH_3	3	7.0
OCH_3	OC_4H_9	4	5.0
OCH_3	OH	4	3.0
OCH_3	OCH_3	2	3.0
OC_2H_5	OC_2H_5	4	1.0
OCH_3	OCH_3	0	0.4
OH	OH	4	0
O-*Sec*butyl	O-*Sec*butyl	4	0
Cl	Cl	4	0

monomethyl tetrachloroterephthalate, and dimethyl dichloroterephthalate. Color intensities decrease in the order given.

Extraction from plant tissues. Recoveries of known amounts of dimethyl tetrachloroterephthalate added to homogenates of cotton seed (*Gossypium* sp.), sweet potatoes (*Ipomoea batatas* Lam.), and onions (*Allium cepa* L.) amounted to 75 to 100 per cent over a range of 0.25 to 3.75 p.p.m. Typical results obtained on fortified samples are shown in Table V. The residues determined thus far varied from less than 0.01 p.p.m. to 1.9 p.p.m. and the majority of the values obtained were under 0.2 p.p.m. In general, the residue content was independent of formulation and rate of application. An extensive residue program is planned during the 1961 crop season and such information with details and modifications of the purification procedure will follow in another paper.

TABLE V

RECOVERY OF DIMETHYL TETRACHLOROTEREPHTHALATE ADDED TO VARIOUS PLANT HOMOGENATES AND SOIL*

Sample material	Added p.p.m.	Found p.p.m.	Recovery %
Cottonseed (nondelinted)	0.25	0.215	86.0
	0.50	0.43	86.0
Cottonseed (delinted)	0.25	0.232	92.8
	0.50	0.518	103.6
Cottonseed oil	1.25	1.10	88.0
	2.50	2.12	84.8
	3.75	3.18	84.8
Onions	0.25	0.235	94.0
	0.50	0.405	81.0
	1.00	0.975	97.5
Sweet potatoes	0.25	0.18	74.0
	0.50	0.41	82.0
	1.00	0.74	74.0
Soil	0.25	0.203	81.2
	0.50	0.414	82.8
	1.00	0.913	91.3
	1.25	1.005	80.4

* All of the above recovery values are means of a minimum of triplicate determinations.

Extraction from soil. Recovery of dimethyl tetrachloroterephthalate from air dry soil when known amounts were added was found to be in the range of 80 to 90 per cent (Table V) when the extraction and purification procedure described under Materials and Methods was followed. In a study in which it was applied to turf at rates of 10 and 20 pounds of active ingredient per acre, periodic samples were taken for one year to determine the residual amount of chemical present. These studies indicated that a

50 per cent wettable powder formulation of dimethyl tetrachloroterephthalate was dissipated from soil more rapidly than a 1.5 per cent granular formulation. After one year 1.8 and 10.3 per cent remained in plots treated at the 10 pound rate and 4.2 and 13.1 per cent remained at the 20 pound rate of the wettable powder and granular formulations, respectively.

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Note

A FIELD METHOD FOR OBSERVATION ON OLFACTORY RESPONSES OF BARK BEETLES (SCOLYTIDAE) TO VOLATILE MATERIALS[1]

J. P. Vité[2] and R. I. Gara[3]

Bark beetles, which depend on the bicambial zone of live host trees, differ in their pattern of host selection from other phytophagous insects in more than one respect. In general, their food-plant range is limited to one host species or to a few species of close botanical relationship. Within their food-plant range only trees of subnormal physiological condition are suitable. Even these trees have to be overcome by mass invasion before the individual insect is able to survive and to breed successfully.

The initial phase of bark beetle attack can be explained upon the random contact and attempted establishment such as are basic to the host-finding processes in many other groups of phytophagous insects (14). Such randomized distribution of beetles cannot, however, explain the subsequent mass invasion when many insects concentrate their attack within a limited period of time on a single host tree. The development of some attractant or other directional force following the initial successful establishment of a single beetle, or relatively few beetles, is the only plausible explanation for the directed locomotion of the beetle population. It seems that this attractant may be a product of a susceptible host tree per se as has been observed in Norway spruce under attack by *Ips typographus* (12). In other coniferous species, it may be the result of the activity of random bark beetles attacking suitable breeding material (2, 15).

Judging from the number of unpublished reports (summarized in 3, 13), efforts to localize the source and to determine the nature of attractants responsible for mass attraction in bark beetle flight have been extensive; several theories have been developed but literature pertaining to critical investigations on olfactory responses of bark beetles is less voluminous (1, 6, 7, 8, 9, 10, 11, 12). Most of the experimental approaches employed laboratory arrangements or olfactometers of various designs. Frequently, the results of these experiments appeared to be contradictory and inconclusive for field application or could not be duplicated. The inadequacy of laboratory approaches to the attraction problem is demonstrated in a continuous search for improvements in existing apparatus and design of new

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[1] These investigations are being conducted under a grant by the National Science Foundation, Washington, D. C.

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instruments. These instruments are of two basic designs or their combinations: the single-choice tube-type (13, p. 45) and the multiple-choice arena-type. Undoubtedly, multiple-choice olfactometers in which the insects change freely from object to object or chamber to chamber must be considered superior to instruments where a single initial left or right orientation is attributed to the preference of the test insects. However, the pitfalls of any olfactometer approach in bark beetle research seem to be twofold: caging effects and the involuntary use of heterogenic or unsuitable test populations such as newly emerged beetles. The arbitrary conditions under which such experiments are performed influence the context of insect behavior to a point where it is difficult to deduce the actual behavior of a population in the field (9, 14).

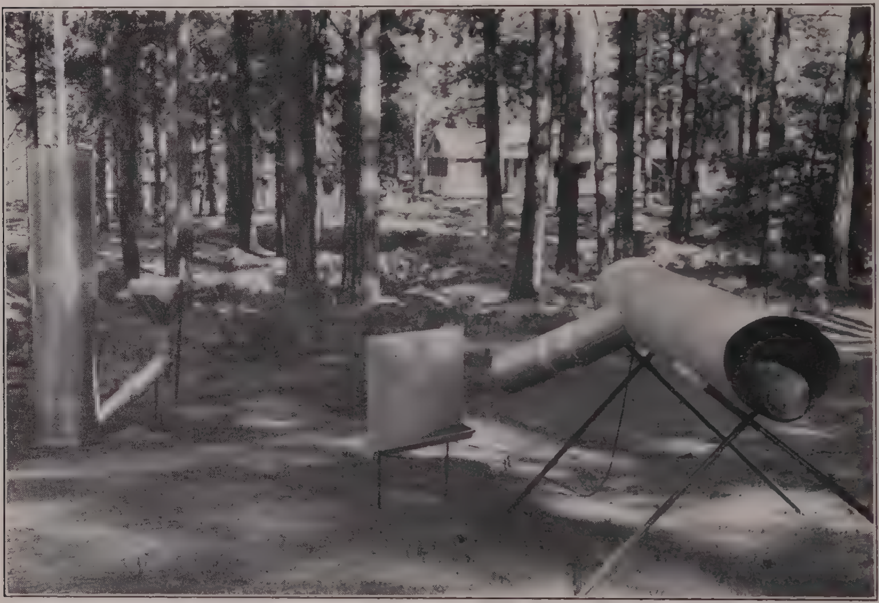

FIGURE 1. Device for trapping bark beetles; an initially infested log producing attractants is placed in the aluminum tube of large diameter. The attractants are blown by a fan against a tangle-foot lined screen, a window glass above a tangle-foot lined paper, or a trough containing water and a mild detergent.

Laboratory attempts to determine materials attractive to *Dendroctonus brevicomis* Lec. and *Ips confusus* (LeConte) by means of y-shaped and arena-type olfactometers were abandoned when a simple field method, where air was blown from cages containing *Ips*-attacked logs, succeeded in attracting *Ips confusus.* In these experiments, aluminum tubes of large diameter were placed in vertical or horizontal position (Fig. 1) as containers of initially infested logs known to be highly attractive (16). Air was drawn by a fan along the infested log and blown against a log or tree to stimulate attack, or against a board or glass for collection of the attracted insects.

A modification of this procedure was used to produce large amounts of attractive material. Stationary incubators or movable chambers (Fig. 2) equipped with a thermostatic heating system were stocked with logs recently infested with bark beetles. Inside these chambers, a small oilless air compressor with the capacity of compressing air up to 30 pounds per square inch circulated the air and forced air from the chamber through a one-eighth inch plastic line of desired length to the point of observation. Forcing the air from large containers through small tubes facilitated the manipulation of air flow by means of flow gauges, filters, and freezing traps. It also permitted the distribution of the air containing the attractant by means of nozzles. The heating system made it possible to stimulate the bark beetle

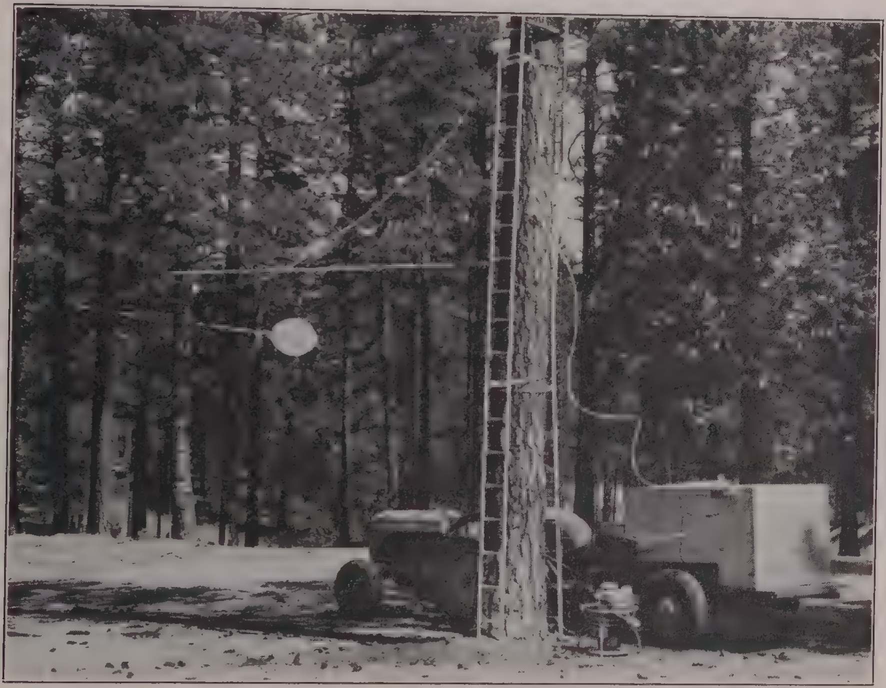

FIGURE 2. Arrangement for testing volatile materials attractive to bark beetles. Attractants are drawn from a transportable box on trailer to the right of tree through plastic tube and exhausted through nozzles installed above a rotary net driven by an electrical motor above ground on extension arm to the left of the tree.

activity inside the chambers, so as to stimulate the production of attractants.

A major concern became the necessity to standardize the observations on the bark beetle population actually in flight and the number of beetles drawn to the release of attractants. A satisfactory sampling method was achieved by the use of rotary nets, a technique originally developed by Chamberlin and Lawson (4) for sampling aerial insect populations. Aerial nets, 38 centimeters in diameter and 70 centimeters deep, affixed to spring steel rings on extendable arms were driven by one-third or one-fourth horsepower electrical motors at a rate of 30 to 120 revolutions per minute. The speed of these nets averaged 54 kilometers per hour sampling an air volume of approximately 6,100 cubic meters per hour. Electrical motors with a reduction gear and high geared motors together with large sized pulleys or fly wheels were equally efficient; motors of less than one-fourth horsepower proved inefficient.

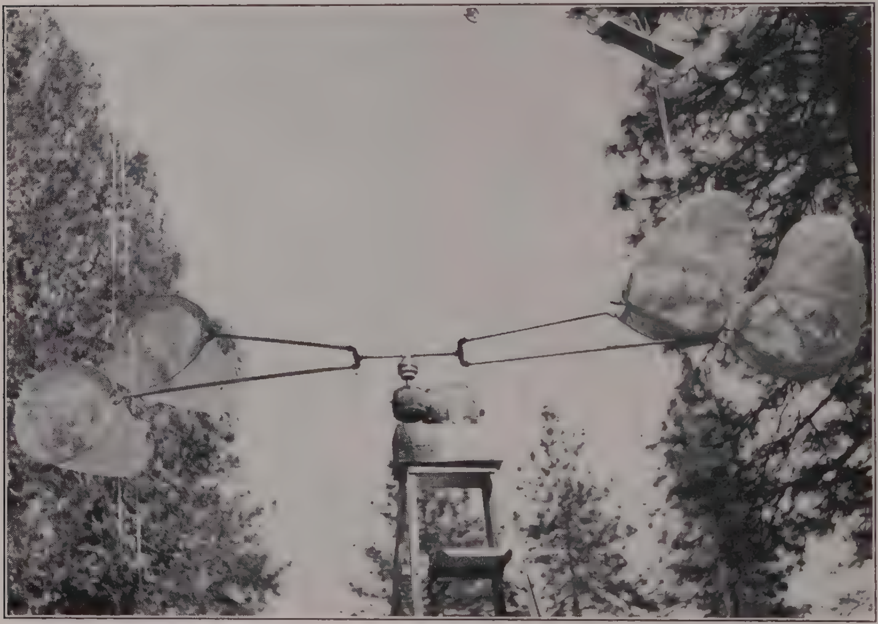

FIGURE 3. Electrical motor with reduction gear designed to drive a combination of four collection nets.

These aerial traps were mounted to trees in either vertical or horizontal positions (Fig. 2) usually 3 meters above the ground, or set on aluminum stepladders (Fig. 3). To avoid unnecessary obstruction of the flight area, the supports sustaining the traps were kept to a minimum.

Preliminary experiments established that one net per trap was more efficient than 2 or more nets (Fig. 3). The most critical factors seemed to be radius and speed with which the aerial net rotated. The efficiency of a window trap and 6 rotary nets with speeds ranging from 20 to 73 kilometers per hour was compared. The result of this study is presented in Figure 4. Nets rotating faster than 43 kilometers per hour were superior to slower moving nets. The efficiency seemed to be best at 53 kilometers per hour in regard to the total number of insects trapped as well as regarding the number of insects per volume sampled. This speed allowed the determination of all Coleoptera caught; however, the more tender insects, especially aphids, were frequently damaged.

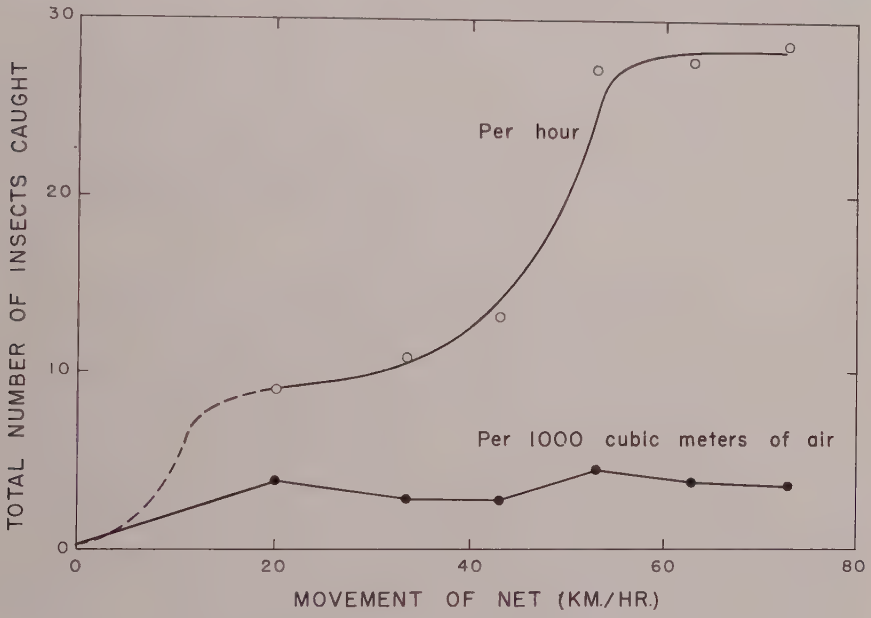

FIGURE 4. The total number of insects of all kinds caught in a series of rotary nets moving at different speeds during a 16-hour test.

The rotary nets proved more efficient than other trapping devices, such as funnels and windows. Even without the use of attractants, the aerial net recorded bark beetle flights continuously while none were caught in funnels. Occasionally, but not consistently, insects were recorded in the window trap. When supplied with attractant, however, the window trap proved useful.

Similar tests were performed with a series of rotary nets with radial arms of various length. As shown in Figure 5, nets rotating in a radius larger than 200 cm. were more efficient than nets describing a smaller arc.

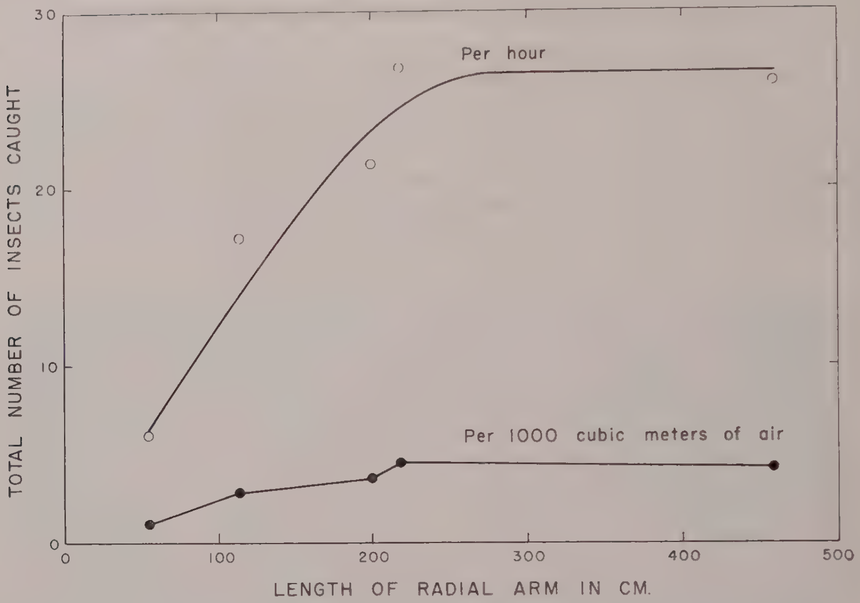

FIGURE 5. The total number of insects of all kinds caught in a series of rotary nets with radial arms of various length during a 12-hour test.

In field studies under epidemic conditions where large numbers of bark beetles and extensive amounts of attractive material are present, the window trap may become more practical because of the vast numbers of insects caught in aerial nets (5).

Results obtained in experiments applying the method described are summarized in Table I. With the onset of bark beetle flight in the Boyce

TABLE I

NUMBER OF BARK BEETLES AND THEIR PREDATORS CAUGHT BY A ROTARY NET WITH AND WITHOUT APPLYING ATTRACTANT IN ALTERNATING PERIODS DURING APRIL 2 AND 4, 1961

Species of beetles collected	Total No. of insects caught in 7½ hrs.		Insects caught per hour	
	Control	Attractant	Control	Attractant
Ips confusus	5	39	0.7	5.4
Ips latidens and *guildi*	3	35	0.4	4.8
Dendroctonus valens	0	10	0	1.4
Other Scolytidae	51	72	7.0	9.6
Ostomidae	0	25	0	3.4
Total	59	181	8.1	24.6

Thompson Institute's Experimental Forest at Grass Valley, California, 16 ponderosa pine logs, 120 cm. long, and 6 to 12 cm. in diameter, approximately 5.4 square meters of bark surface were exposed for 48 hours to the attack of approximately 100 adult *Ips confusus* beetles. The infested logs were then transferred into a chamber and kept there at a temperature between 24° and 28° C. During the following three days of the experiment, tests were made when the outside temperature reached 22° C. The rotary net was alternately operated for equal periods of time (either 30 or 45 minutes) with or without attractants. The attractants were applied through three nozzles installed above the rotary net. To avoid effects of residual attractants, it became necessary to change the net bag after each run in which attractants were used. A total of 59 scolytids were caught during the control tests. However, 156 scolytids were trapped by the same device in the alternate tests using attractants. In addition, 25 Ostomidae known as predators of bark beetles responded to the attractive material.

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SOME RELATIONS BETWEEN THE ORGANIC ACID CONTENT AND THE RESPONSE OF SPINACH TO DIFFERENT PHOTOPERIODS[1]

Edward Harland Buckley[2]

SUMMARY

The content of spinach leaves (*Spinacia oleracea* L. var. Nobel) with respect to L-malic, citric, and succinic acids was studied under conditions of temperature and light which influence flowering. The patterns of quantitative diurnal change for each acid are reported in detail. L-Malic acid exhibited the largest quantitative changes as well as predictable patterns of diurnal variation.

One pattern occurred consistently in vegetative plants, that of malic acid accumulation throughout the photoperiod and depletion during darkness. This pattern is essentially opposite to that found in Crassulacean acid metabolism.

During the photoinduction and early flower bud development there was a sequence of three patterns of diurnal variation. The first resembled that of the vegetative plants and lasted only a few days. The second pattern was characterized by a delay in malate accumulation for the first six to ten hours of the photoperiod (the skotophile period), followed immediately by a very rapid accumulation (the photophile period). The second pattern was induced rapidly and remained only a few days under conditions of rapid and uniform flower induction, but developed slowly and to a lesser degree in populations where flower induction was marginal and uneven. The third pattern resembled the first in that malic acid accumulated from the beginning of the photoperiod. However, maximum accumulation of malate occurred after seven to ten hours of light and net decreases began several hours prior to the dark period.

It is suggested that quantitative changes in malic acid can function as a useful indicator of major metabolic changes associated with the flowering response.

INTRODUCTION

The present state of knowledge pertaining to the flowering response of plants exposed to alternating conditions of light and dark has been reviewed by Lang (14), Liverman (17), and Doorenbos and Wellensiek (7). A number of schemes are discussed which attempt to explain photoperiodism by means of proposed metabolic reactions occurring in the dark and/or light phases of photoperiodic cycles. Although the value of such speculation is obvious, the basic problem of assessing changes in the metabolism of plants resulting from alterations in the photoperiodic regimes still persists. Investigations of the latter type led to the present study.

Gregory *et al.* (9), Spear (26), and Spear and Thimann (27) reported a

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close correlation between the relative rate of dark fixation of carbon dioxide and photoinduction of flowering in *Kalanchoë blossfeldiana* (a short-day plant). Also in soybean and cocklebur (both short-day plants), Langston and Leopold (15) demonstrated the inhibition of flowering through the removal of CO_2 from the atmosphere during the dark. These observations implicated the products of dark CO_2 fixation, such as the carboxylic acids and their metabolic products, with the biochemistry of flowering. Experiments with carbon-14 as $C^{14}O_2$ followed immediately. Unfortunately many compounds in both short-day and long-day plants incorporated appreciable radioactivity during the dark, thereby making interpretation hazardous. However, a consistent pattern was observed for the incorporation of C^{14} into malate. In soybean and cocklebur (short-day plants) radioactivity in malic acid was not detectable after eight hours of darkness in vegetative plants, while malate contained appreciable activity in photoinduced plants. In barley (long-day plant) relatively little radioactivity was incorporated into malic acid during the dark in both vegetative and photoinduced plants, but large amounts were incorporated into malate during the photoperiod in photoinduced plants (24). Barley does not require a long night for flowering, nor does it require CO_2 in the atmosphere at night (15). In *Kalanchoë*, Kunitake *et al.* (13) found a tenfold increase in the dark fixation of C^{14} into malate during photoinduction, but stressed that induced and noninduced leaves contained the same labeled compounds in the same relative amounts.

In these studies with C^{14} the workers were unable to distinguish between equilibrium and nonequilibrium conditions, nor did they reckon with the diurnal fluctuations in the organic acid content of plants. Such considerations led to the detailed quantitative estimation of malic, citric, and succinic acids under flower-inducing and nonflower-inducing photoperiods in the photoperiodically sensitive (long-day) plant, Nobel spinach.

MATERIALS AND METHODS

Seed and seed source. A strain of spinach (*Spinacia oleracea* L. var. Nobel), produced by the W. Atlee Burpee Company, was used in all experiments. Crops produced from seeds of different origin were found to flower either more slowly or nonuniformly or both under the conditions of these experiments.

Experimental design. The seeds were germinated and the plants grown in temperature-controlled rooms in which the temperature was the same as that of the succeeding experimental period. The light exposure (photoperiod) throughout the pre-experimental period was eight hours per day.

The analyses were limited to the leaf rosettes (including the flower spikes when they developed) since Knott (12) demonstrated that the photoperiodic receptors in spinach are located in the leaves.

Uniformity of plant material at the beginning of each experiment was essential. In all cases reported below, the mean dry weight per spinach shoot, excised directly below the leaf rosette, was 0.042 (±0.002) gram at the beginning of the experiment. This criterion of selection ensured both uniformity of starting material and favorable rates of floral induction under a 16-hour photoperiod.

A 16-, 14-, 12-, 11-, 10.5-, 10- or 8-hour photoperiod (starting at 9 a.m.), in a 24-hour cycle, was employed throughout the experimental period and the flowering response was recorded.

The minimum number of 16-hour photoperiods required to initiate floral primordia was also determined and this became the basis for the length of the experimental period employed.

Experiments to determine the average organic acid content for each 24-hour cycle in a particular photoperiodic treatment were accomplished by sampling every 3 hours, then pooling appropriate extracts to obtain composite extracts for analysis.

Experiments to determine the diurnal changes in organic acid content required the analysis of each sample taken every 3 hours. Generally the sampling schedule was 10 a.m., 1 p.m., 4 p.m., 7 p.m. and 10 p.m., 1 a.m., 4 a.m., 7 a.m. and 10 a.m. In terms of the 24-hour cycles (starting with the lights on at 9 a.m.) samples were taken at 1, 4, 7, 10, 13, 16, 19 and 22 hours, and again at 1 hour in the following cycle. When the sampling sequence was otherwise, it is stated in the text and indicated in the figures.

Conditions of growth. The plants were grown in regular greenhouse soil and flats, and were placed in the growth chambers at the time of seeding.

Preliminary investigations were made using a 27°-34° C. temperature range and General Electric (AH 9) mercury arc lighting. Suspended six feet above the plants, the lights provided 3000 f.c. at leaf level. Radiation from this source was predominantly in the blue-violet range. Night temperature was set at 27° C., while temperature during the photoperiod started at 30° C. and increased two to four degrees by the end of the light period.

Subsequent experiments were kept at lower temperatures and a combination of two 40-watt Westinghouse fluorescent tubes per 550-watt incandescent lamp was substituted as a light source. The emitted radiation extended much more evenly throughout the visible range and was higher in the red and far red than in the blue. By suspending the lights five feet above the plant material, an intensity of 1200 f.c. was obtained at leaf level.

Sampling and harvest procedures. Each sample was a composite of plant material collected from four areas in the environment chamber. Usually nine samples were made over a 24-hour period; therefore thirty-six (9×4) sample areas were outlined. Numbered cards were drawn to determine which areas were to be sampled at the designated times. Sampling during the dark period was done in darkness.

The plant material was harvested for assay by cutting directly below the leaf rosette. Fifty grams wet weight of the spinach tops were used for a sample (approximately 100 plants during the first week). Each sample was subdivided into a 10-gram unit for dry weight determination and a 40-gram unit for organic acid analysis. The 40-gram subsamples were homogenized in a VirTis homogenizer for 2 minutes with sufficient ethanol to give a final concentration of 80 per cent.

Extraction, separation, and identification of acids. Prior to analysis, the homogenates were filtered and the residue washed repeatedly with ethanol. The organic acids in the filtrate were separated by Palmer's technique (20) of gradient elution off a Dowex 1 column. A slight modification of the method adapted it to the use of ethanolic rather than aqueous extracts (28).

Tentative identification of the organic acids was made by paper chromatography in 8 solvents listed as b, e, j, k, l, o, q and t by Ranson (21). Crystals of the acids were obtained through the combined use of paper and column chromatography. Identification of the acids was based upon melting points and infrared absorption spectra.

RESULTS

GENERAL MORPHOLOGICAL RESPONSES OF THE PLANTS

The vegetative state was maintained in all experiments by 8-hour photoperiods per day, while flowering was induced as a result of continued 16-hour photoperiods. A 12-hour photoperiod maintained the vegetative state in the high temperature experiment, while it induced flowering at the lower temperatures. In the latter case, the maximum photoperiod for vegetative conditions was 10.5 to 11 hours per day. Table I summarizes the plant response under the three experimental conditions employed.

Limited photoinduction was accomplished at the 27°–34° C. temperature by fifteen cycles of 16-hour photoperiods followed by 12-hour photoperiods. As in the case of continued 16-hour photoperiods (Table I), the flowers appeared 30 to 35 days after the start of photoinduction. The flowering was limited, however, to the few that developed at that time. In all cases this was less than 15 per cent of the population. At the lesser light intensity and temperature conditions, the plants remained vegetative if the last ten to fifteen 16-hour photoperiods prior to flowering were replaced by noninductive photoperiods of 10.5 hours each.

Therefore the apparent minimum time for photoinduction was approximately fifteen days at 27°–34° C., forty days at 24°–26° C., and fifty days at 20°–23° C.

TABLE I

EFFECTS OF PHOTOPERIOD IN THREE TEMPERATURE-LIGHT ENVIRONMENTS UPON THE INITIATION OF STEM ELONGATION AND FLOWERING OF NOBEL SPINACH

Photo period per day, hrs.	No. of days until first stem elongation*			No. of days until first flowering*			Fraction of plants flowering 30 days after first flowering (% total)*		
	20°–23° C.	24°–26° C.	27°–34° C.	20°–23° C.	24°–26° C.	27°–34° C.	20°–23° C.	24°–26° C.	27°–34° C.
8	—	—	—	—	—	—	—	—	—
10			—			—			—
10.5	—	—		—	—		—	—	
11	30–50	40–50		—	—		—	—	
12	40	30	—	70–80	70–80	—	20–60	5–20	—
14			15			30–35			100
16	10	10	15	45–60	40–50	30–35	90	90	100

* Bar indicates no response during experimental period.

ORGANIC ACID COMPOSITION OF THE SHOOT

Succinic, L-malic, citric and fumaric acids were isolated from spinach shoots and crystallized as free acids. Physical methods described above were used to characterize the crystals. Paper chromatography of each acid revealed only one spot which was coincident with that of an authentic specimen. Melting points, mixed melting points, and infrared absorption spectra of the crystals corresponded to those of known samples and therefore proved their identity. Orthophosphoric acid was isolated as a yellowish liquid, and quantitative colorimetric determinations by the method of Berenblum and Chain (3) indicated that it constituted 90 per cent of the phosphate peak.

Oxalic acid, which is a major organic acid in spinach, was poorly extracted and largely volatilized during the evaporation procedure employed.

The order of elution of the acids from the Dowex-1 column is illustrated in Figure 1. L-Malic acid and citric acid were the predominant organic acids observed. Forty to 300 microequivalents of malic acid and 10 to 60 microequivalents of citric acid per gram dry weight were found in spinach shoots. Succinic and fumaric acids were estimated with substantial error. The fumaric acid peak usually could not be resolved unless a slow elution rate from the column was employed.

ESTIMATE OF ANALYTICAL AND SAMPLE ERROR

The initial analyses for the 8-hour, 12-hour, and 16-hour photoperiod

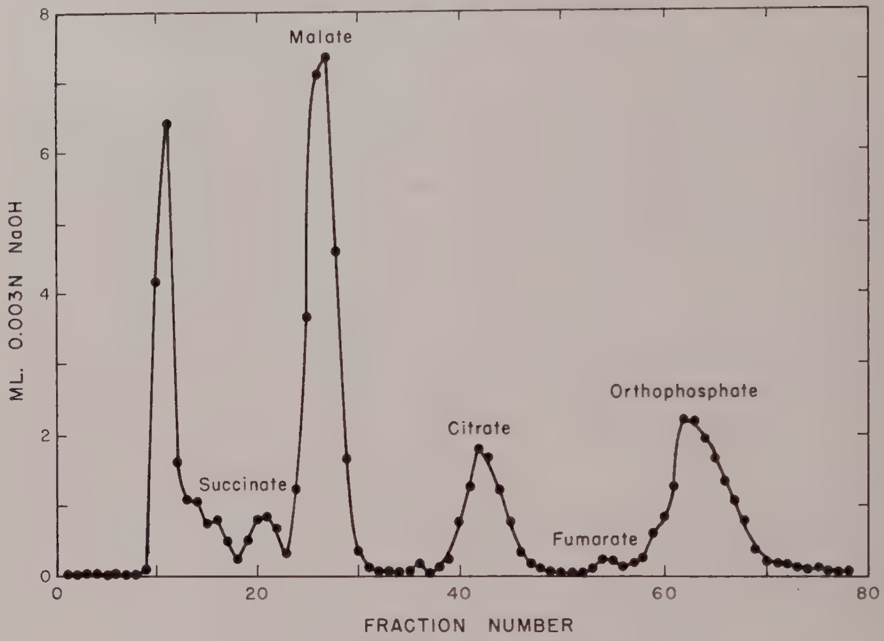

FIGURE 1. Elution pattern of organic acids extracted from Nobel spinach leaves, 0.52 g. dry weight. Fraction size 1.5 ml. to No. 32, 3.0 ml. thereafter.

experiments reported in Figures 2 and 3 constitute triplicate analyses at each designated time since all plant material continued to receive an 8-hour photoperiod during the day. Similarly, analyses made at 1, 4 and 7 hours of the first experimental day may be included to determine statistically the analytical error plus the sample error. From this information the least significant difference at the 5 per cent level with 22 degrees of freedom was calculated for the 20°–23° C. experiment and the 24°–26° C. experiment. A least significant difference of 4.64, 3.01 and 0.93 microequivalents per gram dry weight was found for malate, citrate and succinate, respectively, in the 20°–23° C. experiment, and values of 3.89, 3.05 and 1.02 for malate, citrate and succinate, respectively, in the 24°–26° C. experiment.

CHANGES IN ORGANIC ACID COMPOSITION DURING THE PERIOD OF PHOTOINDUCTION TO FLOWERING

The fluctuating quantities of malic, citric and succinic acids are shown as bar graphs in Figures 2, 3 and 4 in terms of microequivalents per gram dry weight. Stippled bars indicate data from samples taken during the dark period of the cycle. Initial-day, in Figures 2 and 3, is the 24-hour cycle immediately preceding the experimental period.

The correlation between photoinduction and the pattern of diurnal

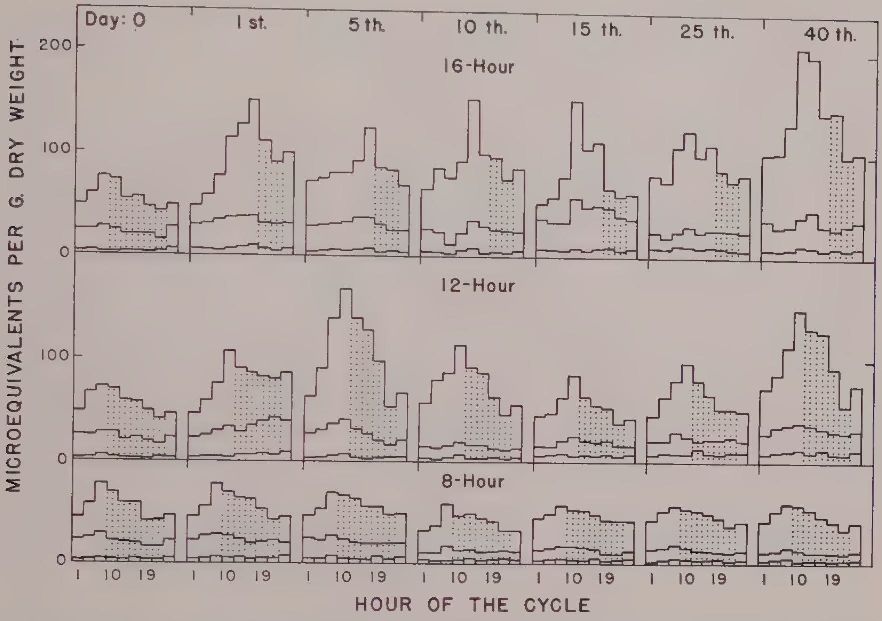

FIGURE 2. Microequivalents of malic, citric and succinic acids present in spinach leaves as influenced by 16-, 12- and 8-hour photoperiods. Temperature 24°–26° C. Malate content is highest, citrate intermediate, and succinate lowest.

quantitative variation of malic acid was first noted in preliminary experiments using mercury arc lighting and 27°–34° C. temperature conditions. The results of these experiments are shown in Figure 5. Plants grown under 8-hour photoperiods were placed under 16-, 14-, 12-, and 8-hour photoperiods. On the fifth day it was observed that plants on a non-photoinductive light cycle (8-hour and 12-hour photoperiods) continued to accumulate malic acid throughout the photoperiod and to lose malic acid during the dark. Subsequently, this is referred to as the vegetative pattern, or the first pattern, of diurnal variation; it is the opposite of that found in Crassulacean metabolism (4, p. 154). However, plants undergoing photoinduction (14-hour and 16-hour photoperiods) lost malic acid during the first seven hours of light. This is referred to as the second pattern of diurnal variation, and, as discussed later, appears to be correlated in time and degree with the assumed amount of photoinduction.

DIURNAL CHANGES IN THE MALIC ACID CONTENT OF VEGETATIVE PLANTS

Photoperiods which maintained the plants in the vegetative state were characterized by only one pattern of diurnal variation; namely, that of malate accumulation throughout the photoperiod, followed by a depletion

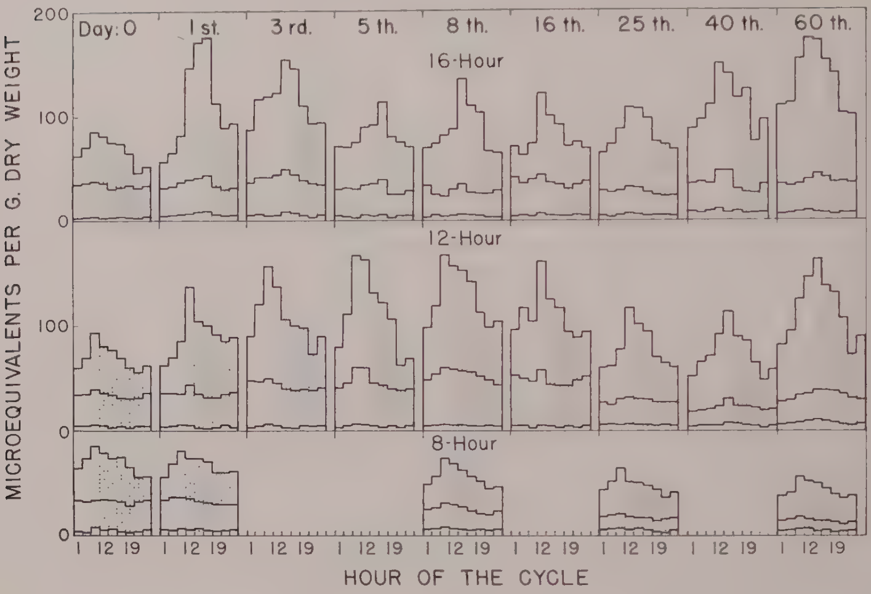

FIGURE 3. Microequivalents of malic, citric and succinic acids present in spinach leaves as influenced by 16-, 12- and 8-hour photoperiods. Temperature 20°–23° C. Malate content is highest, citrate intermediate, and succinate lowest. Analyses made at 1, 4, 8, 12, 14, 16, 19, 22 hours except for 16-hour photoperiod on 25th, 40th and 60th days which were made at 1, 4, 7, 10, 13, 16, 19, and 22 hours.

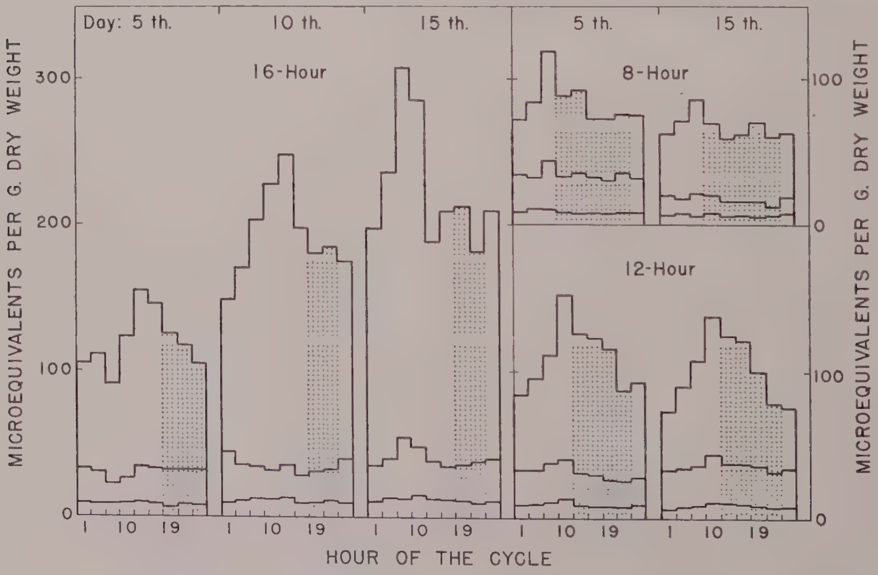

FIGURE 4. Microequivalents of malic, citric and succinic acids present in spinach leaves as influenced by 16-, 12- and 8-hour photoperiods. Temperature 27°–34° C. Malate content is highest, citrate intermediate, and succinate lowest.

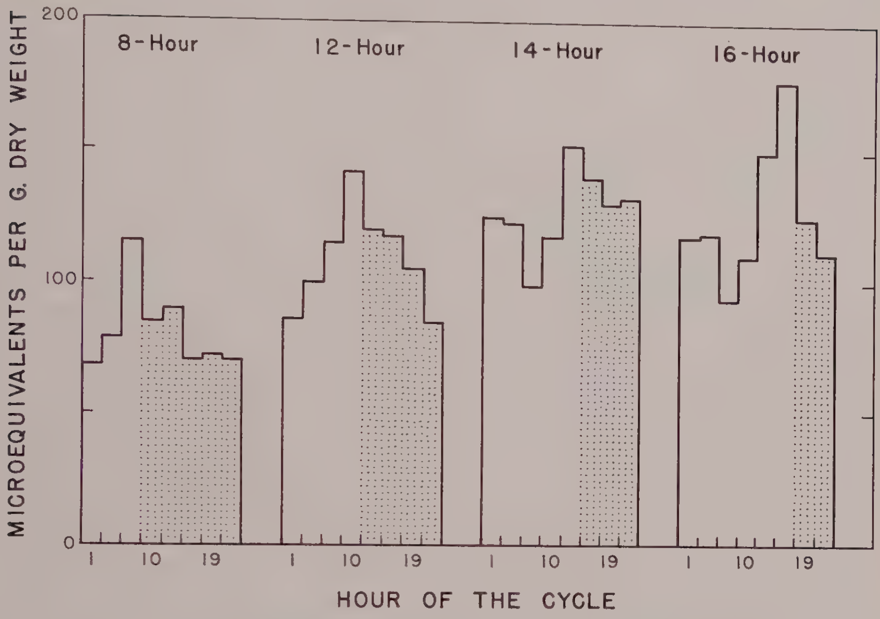

FIGURE 5. Diurnal changes in malic acid content in spinach leaves during the fifth experimental day under vegetative 8-hour and 12-hour photoperiods and under flower-inducing 14-hour and 16-hour photoperiods.

of malate during the dark [Fig. 2 (8-hr.), Fig. 3 (8-hr.) and Fig. 4 (8-hr. and 12-hr.)]. Temperature did not affect the diurnal variation of malate in vegetative plants. The higher concentration of malate observed in the vegetative plants in the 27°–34° C. experiments (Fig. 4) may be due to the different light source and also to the increased duration of light in the case of the 12-hour photoperiods.

DIURNAL CHANGES IN MALIC ACID CONCENTRATION DURING PHOTOINDUCTION

Three basic patterns of diurnal variation were associated with the flower-inducing photoperiods. The first pattern resembled that of the vegetative plants where malate accumulated rapidly throughout most, if not all, of the photoperiod. In the second pattern of diurnal variation the quantity of malate remained fairly constant or decreased during the first six to ten hours of the photoperiod, but then accumulated rapidly during the remaining hours of light. The third pattern of diurnal variation resembled the first in that malate accumulated from the beginning of the photoperiod. However, maximum accumulation was reached after seven to ten hours of light and net decreases began well before the lights were turned off. Only plants on the 16-hour photoperiod reached this stage by the end of the experiment.

All experiments involving a 16-hour photoperiod in a 24-hour cycle resulted in an obvious second pattern of diurnal variation by the fifth experimental day (Fig. 2, 3, and 4). Where flowering was most rapid and uniform (the 27° 34° C. temperature, with mercury arc lights, Fig. 4), the second pattern of diurnal variation not only reached its greatest development, but also disappeared most quickly, i.e., by the tenth day, 20 days before flower buds were visible to the unaided eye. However, where flowering was slower (the lower temperature experiments with fluorescent and incandescent lights, Fig. 2 and 3), the second pattern was still pronounced after the eighth and tenth days, while some indications of it persisted until the fortieth day.

Twelve-hour photoperiods at the lower temperature conditions also induced the second pattern of diurnal variation, but more slowly and to a lesser degree. Only a minor response was observed in the 24° 26° C. experiment (Fig. 2), while a more pronounced second pattern persisted for a longer period in the 20° 23° C. experiment (Fig. 3). By the hundredth day, the flowering response was 14 per cent and 52 per cent, respectively, in these two experiments.

The advantage of the experiments reported in Figures 2 and 3 is that both follow a population through three photoperiodic treatments. The disadvantage is that lack of material did not permit detailed examination of the diurnal changes. This was rectified in part by making analyses of dif-

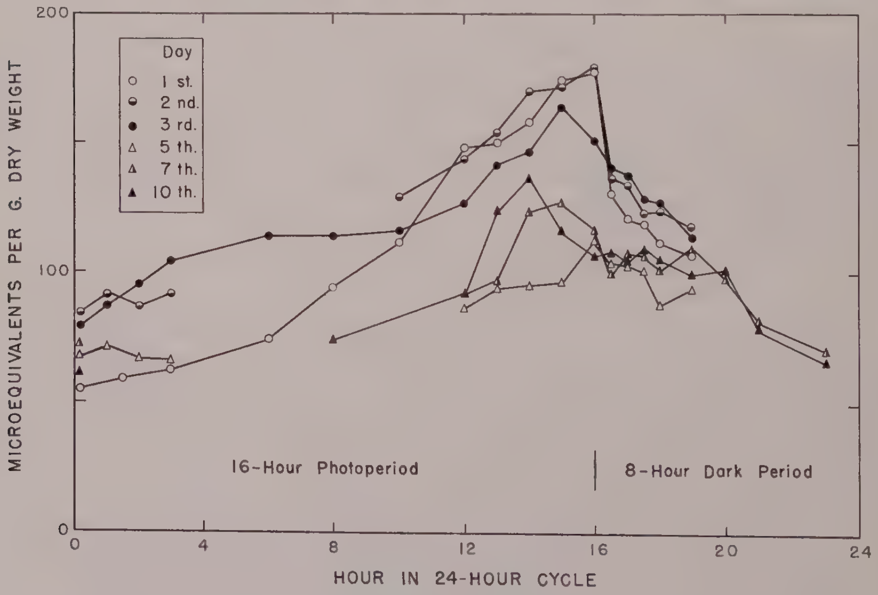

FIGURE 6. Sixteen-hour photoperiod experiments. Detailed changes in malate content of spinach leaves during flower-inducing photoperiods.

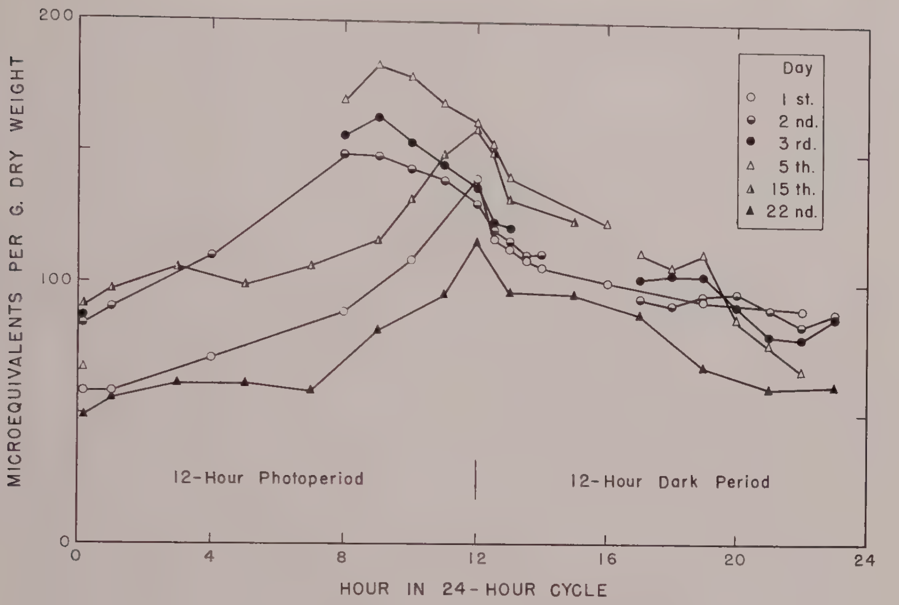

FIGURE 7. Twelve-hour photoperiod experiments. Detailed changes in malate content of spinach leaves during the first 22 flower-inducing photoperiods.

ferent plant populations over short periods of time (a few hours up to 24 hours). Results for malic acid, in experiments within a 20°–24° C. temperature range, are reported in Figure 6 (16-hour photoperiod) and Figure 7 (12-hour photoperiod).

These data show more precisely that malate accumulation in the first 16-hour photoperiod was slow at first (ca. 3 μeq./hr.[3]); then after six hours became moderate (ca. 10 μeq./hr.). At the beginning of the dark period, an extremely rapid drop occurred (ca. 50 μeq. within 30 minutes), followed by a moderate rate of depletion until the beginning of the second day when essentially the same cycle was repeated.

The beginning of the second pattern of diurnal variation was apparent on the third day (Fig. 6). Moderate malate accumulation occurred over the first 4 to 5 hours followed by no change for 5 hours, then continued accumulation at a moderate rate. Usually, however, a process of malate depletion started prior to the dark period and, when this happened, the rate of loss was not altered by the rapid transition from light to dark. On the fifth day there was negligible or slow change in malate content throughout most of the photoperiod, ending with a short period of rapid accumulation. During subsequent days, the period of rapid accumulation (20–30 μeq./hr.) occurred earlier in the day. However, with this came an increasing period of malate depletion during the very last hours of light and then a slight loss of malate during the first four hours of dark, followed by appreciable losses (10-20 μeq./hr.) over the following three hours (Fig. 6). Figures 2 and 3 indicated that the latter pattern of malate depletion in the dark was transitory and was not seen again until the third pattern of diurnal variation was well established.

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[3] Rates of change are per gram dry weight of spinach tops.

The first 12-hour photoperiod (Fig. 7) resembled the first 16-hour photoperiod (Fig. 6) except that the loss of malate at the beginning of the dark period was not as rapid; in fact it essentially stopped after the first two hours. On the second day the pattern changed appreciably. At the beginning of the photoperiod malate accumulated at nearly triple the rate observed on the first day (6-10 μeq. compared to 3 μeq./hr.); then was depleted slowly during the remainder of the photoperiod. Analyses on the third and fifth days also confirmed this period of malate depletion prior to the dark period.

The break in continuity of the data for the second, third and fifth days indicates that other populations were used to obtain the results between the seventeenth and twenty-third hours. During that period of each cycle a transition occurred from negligible change in malate (first day) to marked depletion after the nineteenth hour (third and fifth days).

The second pattern of diurnal variation was well established by the fifteenth and twenty-second days (Fig. 7). However, the delay in malate accumulation was not as pronounced, and lasted only seven to nine hours. The depletion of malate occurred only with the onset of darkness.

DAILY CHANGES IN MALIC ACID CONTENT

The results above indicated that the beginning of the second pattern of diurnal variation resulted in a decrease in the average malate content in a 24-hour cycle. The observation was used to determine the appearance of the second pattern and to compare this with changes in the dry weight of the excised leaf rosettes.

The average quantity of malate per 24-hour cycle (Fig. 8) was obtained under 20°–23° C. temperature conditions for the first 16 experimental days (see Materials and methods). The determination of the dry weight per excised leaf rosette was continued until the sixtieth experimental day. The data in Figure 8 indicate an increase in dry weight due to the longer photoperiods, although the increase for plants on a 16-hour photoperiod is similar to those on a 12-hour photoperiod over the first 16 experimental days. The magnitude of response to a change in photoperiod is indicated by the malate data where the mean malate content drops rapidly from the third to the sixth day under 16-hour photoperiods, but gradually decreases after the seventh day under 12-hour photoperiods.

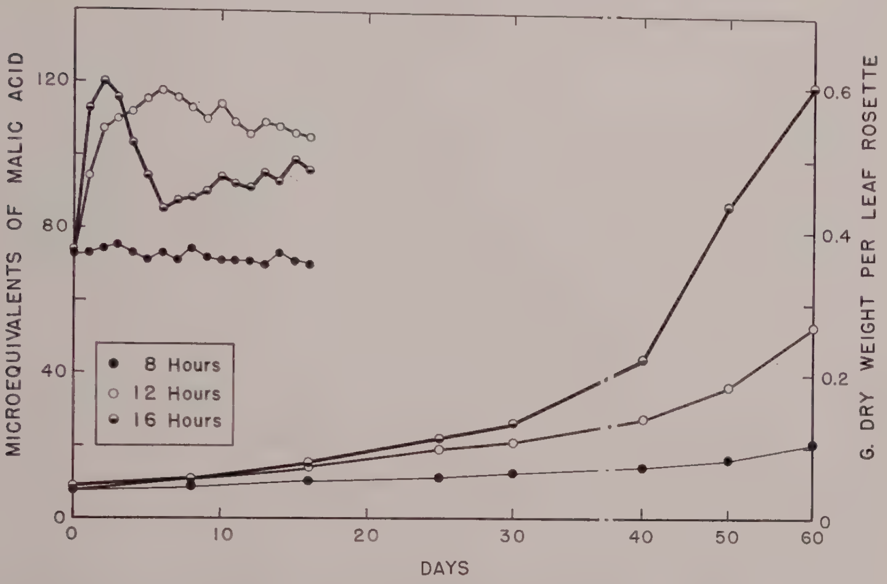

FIGURE 8. The effect on spinach leaves of 16-, 12-, and 8-hour photoperiods on the mean content of malic acid per day and upon the mean dry weight per leaf rosette per day.

CHANGES IN CITRIC ACID CONTENT

The diurnal pattern of citrate content was similar to that of malate in vegetative plants. However, the quantitative changes in citrate during photoinduction were bewildering, since they were not reproducible under apparently similar ecological conditions.

DISCUSSION

The work of Gregory *et al.* (9) was the first refined study to indicate a connection between changes in organic acid metabolism and the processes of photoinduction. They found that *Kalanchoë blossfeldiana*, under photoinductive light cycles, fixed substantial amounts of CO_2 from the atmosphere during the first 15 to 16 hours of dark—the optimum period of dark required for flowering for this short-day species. However, their correlation of CO_2 fixation in the dark with photoinduction was criticized because the pronounced effect occurred after 15 photoinductive cycles, at which time flower primordia were developing. Consequently, the correlation was better with flower development than with flower induction. Whether the type of metabolism which results in floral induction occurs predominantly at the beginning of the photoinductive treatment or later is a matter for debate, but certainly some must occur prior to the formation of flower primordia.

Carbon dioxide fixation in the dark, hence organic acid metabolism, was again implicated in the photoperiodic response of short-day plants by Langston and Leopold (15). They demonstrated that the flowering of Biloxi soybean and *Xanthium* was suppressed by removing atmospheric CO_2 during the dark period. Similar conditions only slightly inhibited the flowering of Winter barley, a long-day plant. Subsequently, Sen and Leopold (24) studied the products of $C^{14}O_2$ fixation during photoinduction. They state (p. 327) that "with respect to the (radioactive) products of dark fixation, it is interesting that malic acid—the major product of dark fixation in a wide variety of plant tissues—is not detectable after 8 hours in vegetative short-day plants (Biloxi soybean and *Xanthium*), but is abundantly present in photoinduced plants. In barley, relatively little (radioactive) organic acids are formed as a consequence of dark fixation, but very large amounts are formed in light. This species does not require CO_2 in the atmosphere at night. Malic acid is particularly abundant after light fixation in barley, and this acid has been found to promote its flowering (16)."

Further work with *Kalanchoë blossfeldiana* has given the best picture of the role of organic acid metabolism. Neyland and Thimann (19) followed the diurnal change in total organic acid content while the plants were maintained on photoperiodic cycles of 8 hours light and 16 hours dark. They found the amounts of organic acid formed corresponded approximately to the amounts of CO_2 previously reported to be fixed by Gregory *et al.* (9). Kunitake *et al.* (13) studied the same response but with $C^{14}O_2$. They found over a tenfold increase in activity in malic acid from photoinduced plants compared with that from vegetative plants, but all compounds that were labeled were similarly affected. Therefore, the short-day photoinductive treatment affects CO_2 fixation in *Kalanchoë* in a strictly quantitative manner. It does not create an additional pathway for CO_2 fixation, but it does alter metabolic rates.

The quantitative data presented in this paper are interpreted also as signs of change in metabolism. The author hopes to investigate these changes, but at present can only suggest possibilities.

The diurnal patterns in malic acid content may be partly attributed to three carboxylation systems. Bandurski and Greiner (2) demonstrated in spinach leaves a very active phosphoenolpyruvate carboxylase which catalyzes the essentially irreversible carboxylation of PEP[4] to form OA and orthophosphate (1). Conn *et al.* (6) found in spinach the malic enzyme, which catalyzes the equilibrium for the reductive carboxylation of pyruvate to malate. Mazelis and Vennesland (18) reported an indication of low phosphoenolypyruvate carboxykinase activity in spinach leaves. It catalyzes the equilibrium between OA and ATP with PEP, ADP and CO_2 (29, 30).

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[4] The following abbreviations are used: PEP, phosphoenolpyruvic acid; OA, oxalacetic acid; PGA, 3-phosphoglyceric acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

The very active PEP carboxylase would be expected to account for most of the malate accumulation in the light. The only control mechanism for this carboxylation appears to be the availability of PEP and CO_2. Therefore, the accumulation of malate during the light is not surprising, since CO_2 would not be a limiting factor (10, 11, 22, 23, 25) and the availability of PEP from PGA during photosynthesis should be ample. The halt in malate accumulation during the latter hours of the photoperiod might indicate a reduction or cessation of photosynthetic CO_2 fixation, resulting in a depletion of PEP.

No explanation is ventured for the delay in malate accumulation which is characteristic of the second pattern of diurnal variation. However, it may represent a major change in metabolism, as indicated in Figure 8. The dry weight over the first eight experimental days increased 0.04 g. under the 8-hour photoperiod, but increased 0.12 g. under both the 12-hour and 16-hour photoperiods. Therefore, the plants with a metabolism represented by the second pattern were unable to utilize the additional four hours of light in terms of an increase in dry weight.

The following correlations indicate that the second pattern of diurnal variation is part of, or is affected by, the metabolism of the flowering response. First, the process of photoinduction must start prior to stem elongation, which is the beginning of the spicate inflorescence under the conditions of these experiments. Stem elongation was observed by the fifteenth day in the 27°–34° C. experiments and by the tenth day in all other experiments with 16-hour photoperiods. The second pattern was well established prior to that time. Under 12-hour photoperiods, at the lower temperatures, the second pattern was obvious by the fifteenth day and stem elongation was observed by the thirtieth or fortieth day; in the high temperature experiment, no second pattern and no stem elongation occurred. Secondly, the degree of flowering response and the uniformity of flowering response could be correlated with the degree of development of the second pattern and the number of days over which it extended. This would be expected from samples composed of populations rather than individuals. Thirdly, Fabian (8) has demonstrated that supplementary low intensity light will promote flowering in long-day plants more readily if used prior to the high intensity light period rather than after it. This observation, along with the dry weight data (Fig. 8) and the second pattern of diurnal variation, would indicate a delay in anabolism which is associated with photoinduction, and occurs at the beginning of the photoperiod. Fourthly, Bünning and Kemmler (5), in their experiments to determine the presence of endogenous rhythms in plants, have shown that *Spinacia* *oleracea* is indifferent, or weakly skotophile, during the first six hours of light, but becomes photophile (receptive to flower induction) during the later hours of the photoperiod. Again, this fits the quantitative changes observed for malic acid in the second pattern of diurnal variation.

ACKNOWLEDGMENTS

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ROLE OF QUINIC ACID IN AROMATIC BIOSYNTHESIS IN HIGHER PLANTS[1]

Leonard H. Weinstein, Clark A. Porter, and
Henry J. Laurencot, Jr.

SUMMARY

Experiments were carried out in which L-quinic-U-C^{14} acid was fed into leaves of a number of higher plants. In general, it was found that labeled quinate was readily metabolized by leaves of Tendergreen beans, Vamorr and Turkish tobacco, McIntosh apple, avocado, *Kalanchoë*, *Rumex acetosa*, corn, oats, and Bonny Best tomato. The major labeled products of all plants studied were always tyrosine, phenylalanine, and $C^{14}O_2$ and often shikimate. In addition, *Kalanchoë*, apple, corn, and Turkish tobacco yielded an unidentified labeled compound, which was found in the free amino acid or protein fraction. This compound was found to be nonreactive to ninhydrin. The broadest spectrum of labeled products was yielded by bean leaves, but most of the radioactivity apparently was derived from dark carboxylations.

The results show conclusively that shikimate, tyrosine, and phenylalanine are preferential products of quinate metabolism in a wide variety of higher plants. The presence of many other labeled products suggests that if the classical aromatic pathway does exist in higher plants, other mechanisms are also operative for the conversion of quinate.

INTRODUCTION

Earlier investigations (17, 18) have shown that L-quinic-C^{14} acid is metabolized by cut rose blooms. The relatively high specific activities found in shikimic acid, tyrosine, and phenylalanine established that L-quinate can be an effective precursor of aromatic compounds in that plant. McCalla and Neish (12) and Gamborg and Neish (7) have shown that shikimate is readily converted to tyrosine and phenylalanine in *Salvia*, buckwheat, and wheat plants, but evidence that quinate is a precursor to shikimate synthesis is not available. The enzymes required to convert 5-dehydroquinate→5-dehydroshikimate→shikimate have been demonstrated (13, 19) and purified (3, 4) from higher plants. Quinic dehydrogenase, the enzyme responsible for the conversion of quinate→5-dehydroquinate has not been demonstrated from a higher plant source.

The rose plant and other members of the Rosaceae (1, 2, 5, 8, 9, 10, 11, 16, 17, 18) are rich in quinic acid. Since the metabolic pathway of quinate in the rose is fairly specifically oriented to formation of the aromatic amino

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acids, the studies were expanded to include 10 additional species or varieties of plants, representing 7 plant families. With the exception of apple, none of the species represented contains a high level of endogenous quinate.

Sprinson (15) and Neish (14) have recently published excellent reviews of aromatic biosynthesis.

MATERIALS AND METHODS

Experiments with young leaves of Tendergreen bean (*Phaseolus vulgaris* L.), McIntosh apple (*Pyrus malus* L.), *Kalanchoë* sp., Vamorr 48 tobacco (*Nicotiana tabacum* L.), and avocado (*Persea americana* Mill.) were conducted using L-quinate-U-C^{14} having a specific activity of about 77,200 c.p.m./mg. Later experiments with leaves of Turkish tobacco (*Nicotiana tabacum* L.), McIntosh apple (*Pyrus malus* L.), sorrel (*Rumex acetosa* L.), Tendergreen bean (*Phaseolus vulgaris* L.), Bonny Best tomato (*Lycopersicon esculentum* L.), and *Kalanchoë* sp., and seedlings of corn (*Zea mays* L.) and oat (*Avena sativa* L.) utilized L-quinate-U-C^{14} having a specific activity of about 1,200,000 c.p.m./mg. (as counted on a D-47 gas-flow head with a micromil window). Prior to feeding the quinate into the leaves, it was neutralized with NaOH and diluted to an appropriate volume with 0.05*M* phosphate buffer, pH 6.8. Detached leaves of the above plants were allowed to absorb 5 mg. of the lower specific activity quinate or 1 mg. of the higher specific activity quinate in the dark. After absorption the leaves were placed in water and kept in the dark for 48 hours, during which time the CO_2 released was continuously trapped in NaOH. At the end of the 48-hour period, the leaves were killed and extracted with 80 per cent ethanol. Analyses were carried out as described previously (18), except that in the later experiment radioactivity of free and bound amino acids was determined by direct counting of 2-dimensional paper chromatograms.

RESULTS

The results presented herein represent data from two of several experiments. Results of experiments using low specific activity quinate are shown in Tables I to V. It can be generally stated that labeled quinate is readily metabolized by the plant species used with 59.9, 27.9, 15.7, 14.0, and 12.9 per cent of the quinate absorbed being utilized in leaves of beans, tobacco, apple, avocado, and *Kalanchoë*, respectively. The distribution of labeled compounds derived from quinate is fairly wide, especially in bean, tobacco, and apple leaves. Some radioactivity is usually present in the Krebs cycle acids and their corresponding amino acids. The label in these compounds appears to be due to dark fixation following $C^{14}O_2$ production. Ample quantities of $C^{14}O_2$ are produced as can be seen in the tables with the greatest percentage being evolved by *Kalanchoë*, followed by tobacco, avocado, apple, and bean.

TABLE I

DISTRIBUTION OF C^{14} IN TENDERGREEN BEAN LEAVES FED L-QUINIC-U-C^{14} ACID
(Specific activity 77,200 c.p.m./mg.)

Fraction	C.p.m. as % of toal C^{14} in fraction*	C.p.m. as % of total C^{14} absorbed**
Respiratory CO_2	100.0	1.7
Organic Acids		
Unknown A	5.1	4.2
Unknown B	0.7	0.6
Unknown C	1.0	0.8
Pyrrolidone carboxylic	1.4	1.2
Shikimic	89.5	74.8
Malic	1.7	1.4
Tartaric	0.2	0.1
Malonic	0.2	0.2
Fumaric	0.3	0.2
Free Amino Acids and Amides		
Aspartic	1.4	0.1
Asparagine	5.0	0.3
Glutamic	1.7	0.1
Alanine	1.6	0.1
Tyrosine	52.0	2.9
Phenylalanine	38.2	2.1
Protein Amino Acids		
Tyrosine	60.5	1.1
Phenylalanine	39.5	0.7
Neutral Fraction	100.0	0.7
Humin	100.0	6.6

* Does not include quinic acid.
** Per cent conversion to shikimate, tyrosine, and phenylalanine, and the per cent of quinate utilized were 81.6 and 59.9, respectively.

In the organic acid fraction of bean (Table I), the major labeled constituent was shikimate, accounting for 89.5 per cent of the radioactivity in that fraction. Likewise, in the free amino acid and protein amino acid fractions, the aromatic amino acids, tyrosine and phenylalanine, accounted for 52.0 and 38.2 and 60.5 and 39.5 per cent of the radioactivity, respectively. Of the total amount of quinate absorbed into bean leaves, 81.6 per cent was recovered as products or intermediates in aromatic metabolism.

The distribution of labeled products of quinate metabolism in Vamorr tobacco was very broad, especially in the free amino acid fraction (Table II). Shikimate accounted for 44.7 per cent of the radioactivity in the organic acid pool, while tyrosine and phenylalanine made up 20.2 and 33.3 and 17.9 and 54.2 per cent, respectively, of the free amino acid and protein fractions. A total of 40.1 per cent of the total quinate absorbed was recovered in shikimate, tyrosine, and phenylalanine.

Results for apple leaves (Table III) followed the same general pattern, with shikimate representing 81.1 per cent of the organic acids and tyrosine and phenylalanine 15.5 and 32.0 and 15.1 and 40.2 per cent, respectively, of the free and protein amino acids. In the free amino acid pool, however, an unknown compound, not reactive with ninhydrin, had the highest radioactivity. A total of 49.0 per cent of the total quinate absorbed was accounted for by aromatic biosynthesis.

No labeled shikimate was detected in avocado leaves (Table IV) and the dominant labeled compound is presumed, on the basis of column and paper chromatographic evidence, to be α-ketoglutarate. Most of the labeled tyrosine and phenylalanine was incorporated into protein. These compounds represented a total of 15.2 per cent of the total C^{14} absorbed by avocado leaves.

TABLE II

DISTRIBUTION OF C^{14} IN VAMORR TOBACCO LEAVES FED L-QUINIC-U-C^{14} ACID
(Specific activity 77,200 c.p.m./mg.)

Fraction	C.p.m. as % of total C^{14} in fraction*	C.p.m. as % of total C^{14} absorbed**
Respiratory CO_2	100.0	13.9
Organic Acids		
Shikimic	44.7	11.2
Malic	24.4	6.1
Succinic	4.0	1.0
Citric	18.9	4.7
Fumaric	8.0	2.0
Free Amino Acids and Amides		
Aspartic	3.6	0.9
Asparagine	6.7	1.7
Alanine	7.1	1.7
Cysteic	4.4	1.1
Glutamic	1.5	0.4
Glutamine	4.0	1.0
Glycine	1.1	0.3
Histidine	4.6	1.1
Lysine	4.1	1.0
Serine	5.4	1.3
Valine	4.1	1.0
Tyrosine	20.2	5.0
Phenylalanine	33.3	8.2
Protein Amino Acids		
Alanine	11.0	2.4
Aspartic	8.2	1.8
Glutamic	8.8	1.9
Tyrosine	17.9	3.9
Phenylalanine	54.2	11.8
Neutral Fraction	100.0	7.5
Humin	100.0	7.1

* Does not include quinic acid.

** Per cent conversion to shikimate, tyrosine, and phenylalanine, and the per cent of quinate utilized were 40.1 and 27.9, respectively.

TABLE III

DISTRIBUTION OF C^{14} IN MCINTOSH APPLE LEAVES FED L-QUINIC-U-C^{14} ACID
(Specific activity 77,200 c.p.m./mg.)

Fraction	C.p.m. as % of total C^{14} in fraction*	C.p.m. as % of total C^{14} absorbed**
Respiratory CO_2	100.0	3.7
Organic Acids		
Shikimic	81.1	32.5
Malic	10.2	4.1
Citric	8.7	3.5
Free Amino Acids and Amides		
Alanine	10.4	1.9
Aspartic	6.7	1.2
Asparagine	2.8	0.5
Glutamic	32.7	5.9
Tyrosine	15.5	2.8
Phenylalanine	32.0	5.7
Protein Amino Acids		
Alanine	13.0	1.9
Aspartic	5.0	0.7
Leucines	13.1	1.9
Lysine	13.5	1.9
Tyrosine	15.1	2.2
Phenylalanine	40.2	5.8
Neutral Fraction	100.0	13.8
Humin	100.0	10.1

* Does not include quinic acid.

** Per cent conversion to shikimate, tyrosine, and phenylalanine, and the per cent of quinate utilized were 49.0 and 15.7, respectively.

Metabolism of quinate by *Kalanchoë* leaves followed the usual pattern in that shikimate and the aromatic amino acids were labeled. The major labeled compound, however, was found to be a ninhydrin-negative constituent of the protein amino acid fraction. Later studies with *Kalanchoë* and other plants have confirmed this, except that it has often been found in the free amino acid fraction also. Since the amino acid fractions were taken up on Dowex 50, hydrogen-form, the unknown compound is cationic.

In the experiments reported here, in both bean and *Kalanchoë* leaves, tyrosine was more preferentially labeled than phenylalanine. The reverse was true in tobacco, apple, and avocado leaves. This was not always the case, however. In a similar experiment with Tendergreen bean, Vamorr tobacco, McIntosh apple, Bonny Best tomato, and *Rumex acetosa*, phenylalanine was always more radioactive than tyrosine. The values for phenylalanine and tyrosine in the order of plant species listed above were 58.0 and 12.5 per cent, 57.5 and 32.0 per cent, 66.3 and 13.6 per cent, 66.0 and 8.8 per cent, and 66.7 and 22.4 per cent of the total label in free and protein amino acids.

TABLE IV

DISTRIBUTION OF C^{14} IN AVOCADO LEAVES FED L-QUINIC-U-C^{14} ACID
(Specific activity 77,200 c.p.m./mg.)

Fraction	C.p.m. as % of total C^{14} in fraction*	C.p.m. as % of total C^{14} absorbed**
Respiratory CO_2	100.0	5.6
Organic Acids		
Malic	15.0	5.1
Citric	10.6	3.6
α-Ketoglutaric	74.4	25.1
Free Amino Acids and Amides		
Alanine	30.2	1.1
Aspartic	7.5	0.3
Asparagine	3.2	0.1
Glutamic	8.5	0.3
Tyrosine	17.2	0.6
Phenylalanine	33.4	1.2
Protein Amino Acids		
Alanine	12.3	3.2
Aspartic, glutamic	26.4	6.8
Leucines	3.8	1.0
Valine	5.3	1.4
Tyrosine	18.0	4.6
Phenylalanine	34.3	8.8
Neutral Fraction	100.0	20.9
Humin	100.0	10.2

* Does not include quinic acid.

** Per cent conversion to shikimate, tyrosine, and phenylalanine, and the per cent of quinate utilized were 15.2 and 14.0, respectively.

In this later experiment it was possible to determine the specific activity of nonvolatile organic acids. Data for both bean and *Rumex* are shown in Table VI.

Later experiments were run principally to determine accurately the conversion of quinate to aromatic amino acids. High specific activity quinate (1,200,000 c.p.m./mg.) was used in order that amino acids could be identified by autoradiography. Five grams of the following tissues were used in these experiments: young leaves of Tendergreen bean, *Rumex acetosa*, *Kalanchoë* sp., Bonny Best tomato, McIntosh apple, Turkish tobacco, and young seedlings of oat and corn.

Figure 1 shows curves of $C^{14}O_2$ evolution during the 48-hour dark incubation period. Bean leaves evolved nearly 48 per cent of the total C^{14}-labeled quinate supplied as $C^{14}O_2$; *Rumex* and *Kalanchoë* evolved about 22 per cent; corn and oat about 15 per cent; and tomato, apple, and tobacco about 8 per cent. It is likely that at least a part of the CO_2 production occurs from decarboxylation of prephenic acid to phenylpyruvic acid in

TABLE V

DISTRIBUTION OF C^{14} IN KALANCHOË LEAVES FED L-QUINIC U-C^{14} ACID
(Specific activity 77,200 c.p.m./mg.)

Fraction	C.p.m. as % of total C^{14} in fraction*	C.p.m. as % of total C^{14} absorbed**
Respiratory CO_2	100.0	24.2
Organic Acids		
Shikimic	85.6	17.2
Malic	10.7	2.1
Isocitric, citric	3.7	0.7
Free Amino Acids and Amides		
Tyrosine	79.7	3.9
Phenylalanine	20.3	1.0
Protein Amino Acids		
Unknown	62.7	20.2
Glutamic	11.7	3.8
Tyrosine	13.6	4.4
Phenylalanine	12.0	3.9
Neutral Fraction	100.0	5.0
Humin	100.0	13.7

* Does not include quinic acid.

** Per cent conversion to shikimate, tyrosine, and phenylalanine, and the per cent of quinate utilized were 30.4 and 12.9, respectively.

TABLE VI

SPECIFIC ACTIVITIES OF NONVOLATILE ORGANIC ACIDS OF LEAVES OF TENDERGREEN BEAN AND RUMEX ACETOSA FED L-QUINIC-U-C^{14} ACID
(Specific activity 77,200 c.p.m./mg.)

Organic acid	Specific activity (c.p.m./mg.)
Bean	
Shikimic	8,150
Quinic	8,550
Pyrrolidone carboxylic	145
Succinic	120
Malic	130
Malonic	50
Fumaric	190
Rumex	
Shikimic	11,450
Quinic	10,700
Pyrrolidone carboxylic	224
Malic	15
Tartaric	25
Citric	80

the pathway to the aromatic amino acids (15). However, more $C^{14}O_2$ was evolved than can be accounted for by conversion to phenylalanine and tyrosine (Fig. 1, Tables VII and VIII). The remainder is due to an unknown mechanism.

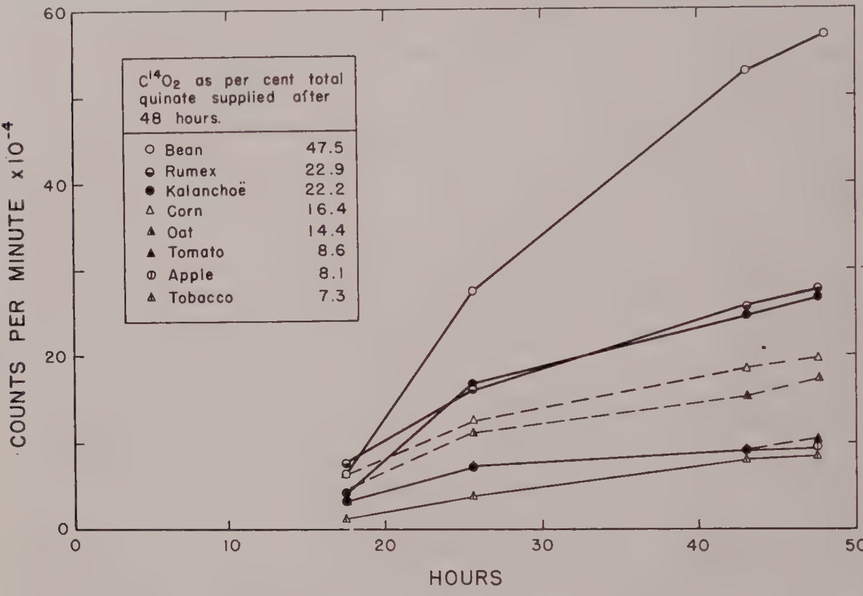

Figure 1. Evolution of $C^{14}O_2$ from leaves supplied with L-quinic-U-C^{14} (Sp. act. 1,200,000 c.p.m./mg.).

Bean leaves converted quinate to the broadest spectrum of free amino acids of the plants studied (Table VII and Fig. 2 A). With the exception of tryptophan, the identity of which is somewhat questionable, and the aromatic amino acids, the remaining labeled acids were probably formed via dark fixation of $C^{14}O_2$. This has been strongly indicated by degradation of malic acid in which all radioactivity was shown to be in carbons 1 and 4. Tobacco, *Rumex*, tomato, oat, and corn (Table VII and Fig. 2 B, C, D, E, F) all resulted in production of labeled amino acids related to the Krebs cycle. The relatively high activity of valine in tomato was an exception. The dominant unknown compound present in the free amino acid fraction of apple and *Kalanchoë* was not reactive to ninhydrin. Its position in phenol and *n*-butanol-acetic acid—water lay just above, but was distinctly separate from glutamic acid.

In all plants except *Kalanchoë* the combined total radioactivity in tyrosine and phenylalanine represented from 55.7 per cent (apple) to 97.2 per cent (tobacco) of the total activity in free amino acids. On the basis of total counts, tyrosine was more radioactive than phenylalanine in

TABLE VII

DISTRIBUTION OF C^{14} AND SPECIFIC ACTIVITY OF FREE AMINO ACIDS OF LEAVES FED L-QUINIC-U-C^{14} ACID
(Specific activity 1,200,000 c.p.m./mg.)

Species	Total c.p.m.	% Of total free amino acids	Specific activity (c.p.m./mg.)
Bean			
Aspartic	3,410	2.9	3,760
Asparagine	19,730	16.8	2,330
Glutamic	3,380	2.9	4,385
Glutamine	1,725	1.5	3,290
γ-Aminobutyric	1,690	1.4	3,830
Tryptophan	2,210	1.9	—
Tyrosine	56,000	47.7	273,170
Phenylalanine	29,300	25.0	39,605
Tobacco			
Asparagine	1,240	2.8	480
Tyrosine	11,930	27.3	61,975
Phenylalanine	30,650	69.9	71,395
Apple			
Unknown*	1,450	44.3	—
Tyrosine	829	25.2	20,730
Phenylalanine	1,000	30.5	25,000
Rumex			
Aspartic	2,175	3.6	8,790
Glutamic	2,345	3.9	2,695
Glutamine	3,765	6.2	3,190
Tyrosine	34,250	56.6	244,640
Phenylalanine	17,980	29.7	58,320
Tomato			
Asparagine	1,900	1.4	500
Glutamine	1,795	1.3	1,500
Valine	7,180	5.2	5,555
Tyrosine	55,000	39.6	160,580
Phenylalanine	72,900	52.5	87,485
Oat			
Asparagine	5,630	11.4	875
Glutamine	1,000	2.0	810
Tyrosine	19,860	40.2	33,805
Phenylalanine	22,900	46.4	12,225
Corn			
Alanine	2,900	2.6	540
Asparagine	6,700	6.1	860
Tyrosine	77,700	70.3	135,090
Phenylalanine	23,150	21.0	15,850
Kalanchoë			
Unknown*	7,140	85.2	—
Tyrosine	310	3.7	3,875
Phenylalanine	930	11.1	2,940

* Does not react with ninhydrin.

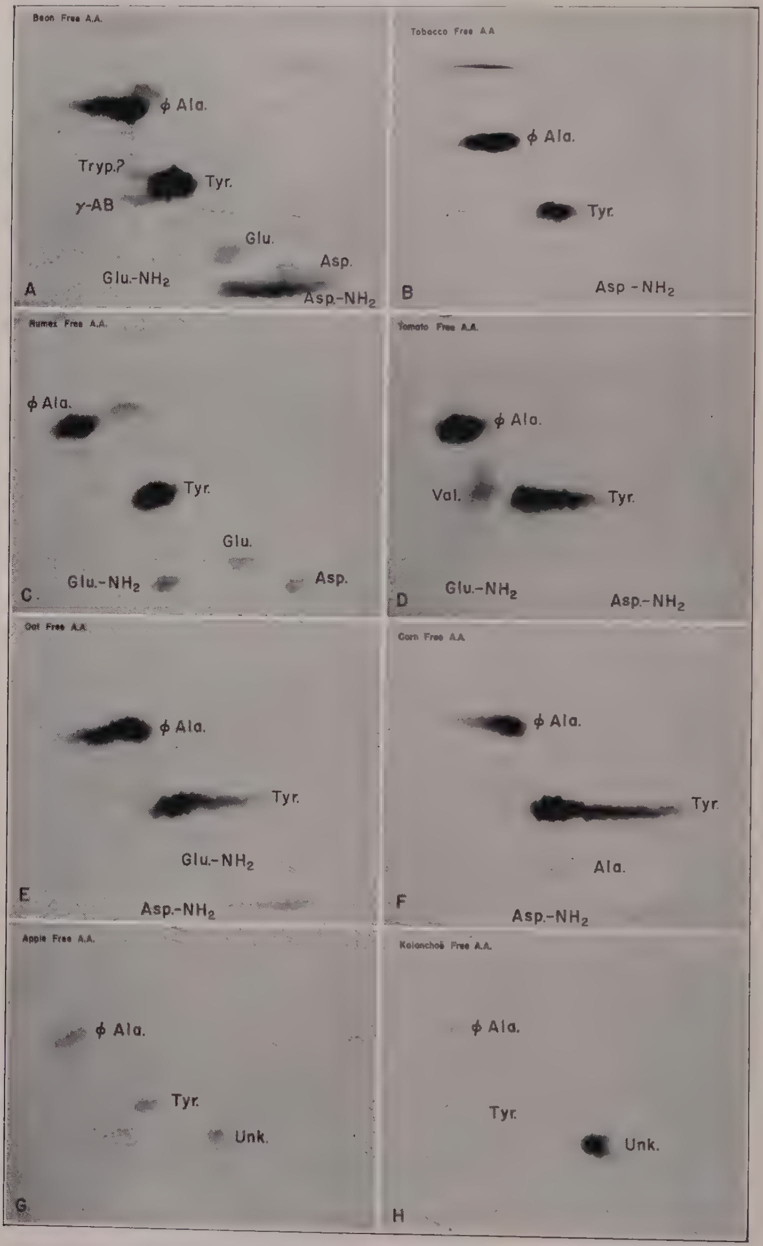

FIGURE 2. Autoradiograms of free amino acids of leaves of plants fed L-quinic-U-C^{14} acid: (A) Tendergreen bean, (B) Turkish tobacco, (C) *Rumex acetosa*, (D) Bonny Best tomato, (E) oat, (F) corn, (G) McIntosh apple, and (H) *Kalanchoë* sp. ϕ Ala = Phenylalanine; Tyr = tyrosine; Tryp = tryptophan; γ-AB = *gamma*-aminobutyric acid; Glu = glutamic acid; Glu-NH$_2$ = glutamine; Asp = aspartic acid; Asp-NH$_2$ = asparagine; Ala = alanine; Val = valine; Unk = unknown.

bean, *Rumex*, and corn (Table VII). On the basis of specific activity, however, tyrosine was more radioactive in bean, *Rumex*, tomato, oat, corn, and *Kalanchoë*.

The distribution of C^{14} from quinate in the protein amino acids was very limited. The ninhydrin-negative compound found in the free amino acid fraction of apple and *Kalanchoë* was also present in the protein of tobacco and corn (Table VIII and Fig. 3 B and F). In neither case, however, was it the dominant compound. The only radioactive amino compound found in protein other than the aromatic amino acids was glutamic acid in *Rumex* protein (Table VIII and Fig. 3 C). No radioactivity was detectable in *Kalanchoë* protein amino acids by autoradiography. Tyrosine and phenylalanine represented from 90.5 to 100 per cent of the total label in protein amino acid. On the basis of total counts, tyrosine was more radioactive than phenylalanine in bean, *Rumex*, and corn (Table VIII).

TABLE VIII

DISTRIBUTION OF C^{14} AND SPECIFIC ACTIVITY OF PROTEIN AMINO ACIDS OF LEAVES FED L-QUINIC-U-C^{14} ACID

(Specific activity 1,200,000 c.p.m/mg.)

Species	Total c.p.m.	% of total protein amino acids	Specific activity (c.p.m./mg.)
Bean			
Tyrosine	96,250	53.6	46,400
Phenylalanine	83,200	46.4	19,030
Tobacco			
Unknown*	6,210	9.5	—
Tyrosine	13,870	21.3	14,000
Phenylalanine	45,200	69.2	17,630
Apple			
Tyrosine	21,600	35.3	6,650
Phenylalanine	39,500	64.7	6,980
Rumex			
Glutamic	4,850	5.3	1,100
Tyrosine	70,100	76.2	126,600
Phenylalanine	17,050	18.5	11,630
Tomato			
Tyrosine	86,500	49.9	52,000
Phenylalanine	86,750	50.1	20,700
Oat			
Tyrosine	16,150	49.2	12,380
Phenylalanine	16,720	50.8	6,730
Corn			
Unknown*	1,480	1.8	—
Tyrosine	49,900	62.2	10,680
Phenylalanine	28,850	36.0	2,850

* Does not react with ninhydrin.

On the basis of specific activity, tyrosine was more radioactive in bean, *Rumex*, tomato, oat, and corn. The results based on total counts and specific activity were the same as those found for free amino acids. In tomato and oat, however, the total counts in the two amino acids were the same.

The total activity of tyrosine and phenylalanine was greater in protein

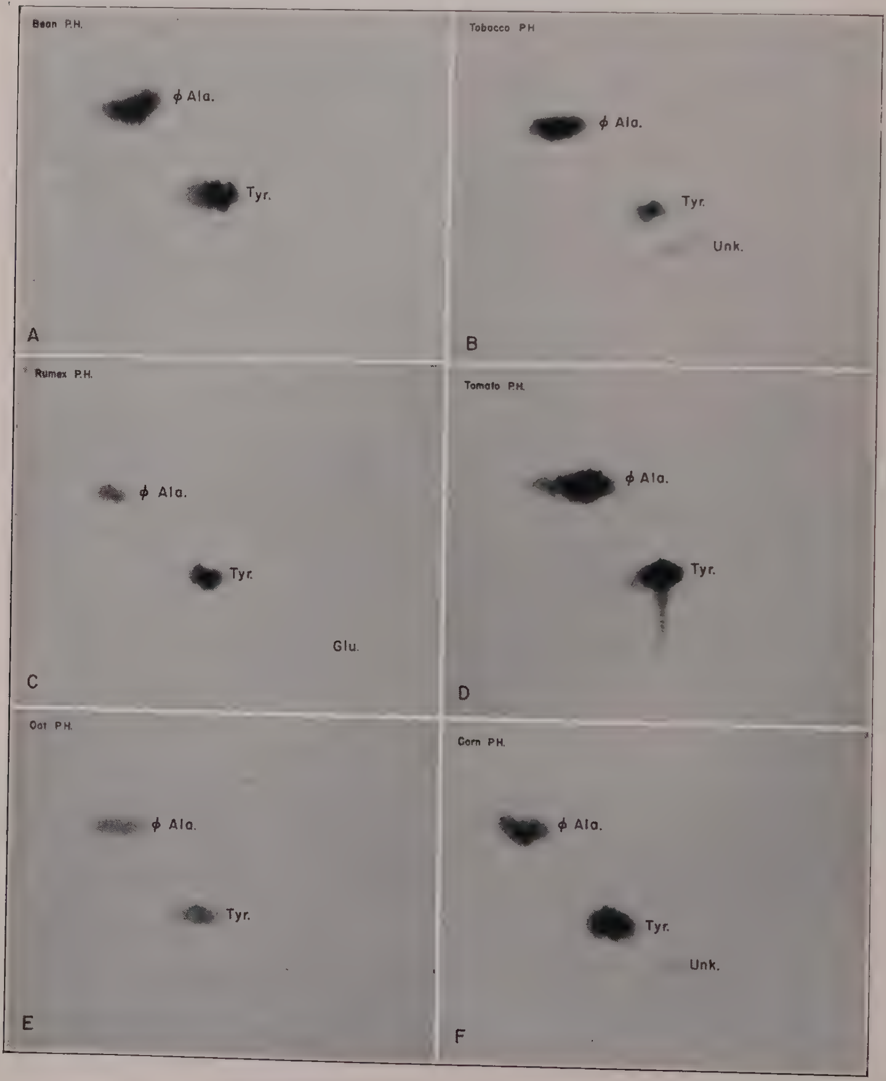

FIGURE 3. Autoradiograms of protein amino acids of leaves of plants fed L-quinic-U-C^{14} acid: (A) Tendergreen bean, (B) Turkish tobacco, (C) *Rumex acetosa*, (D) Bonny Best tomato, (E) oat, and (F) corn. Autoradiogram of apple protein amino acids similar to that of oats. (See Fig. 2 for abbreviations.)

amino acids than in free amino acids in all species except corn, oat, and *Kalanchoë* (Tables VII and VIII).

DISCUSSION

It is apparent from the results that exogenously added quinic acid is readily converted to aromatic amino acids or their precursors in many higher plants. This is true even in leaves of plants from which endogenous quinic acid has not been reported (*Kalanchoë* and avocado). In every instance, the specific activities of tyrosine and phenylalanine are enormously higher than those of other labeled amino acids (Tables VII and VIII). It is also obvious that other pathways for quinic acid metabolism must also exist in each of the plants studied. This is shown most clearly by the relatively high amount of respiratory $C^{14}O_2$, and the rather broad spectrum of labeled products found.

The results reported herein provide good evidence that the pathway by which quinate is converted to shikimate and aromatic amino acids is similar to the classical microbial pathway (6). However, no evidence has yet been forthcoming from these studies that intermediate products between quinate and shikimate, such as 5-dehydroquinate and 5-dehydroshikimate, exist in higher plants fed quinate or which are rich in endogenous quinate. Some additional evidence, however, that the aromatic pathway in higher plants is not similar to that of *E. coli* and *Neurospora* mutants is derived from recent unpublished studies showing that very little radioactivity from labeled quinate is found in tryptophan of beans.

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EFFECTS OF ATMOSPHERIC FLUORIDE ON METABOLIC CONSTITUENTS OF TOMATO AND BEAN LEAVES

Leonard H. Weinstein

SUMMARY

Time-course experiments were run in order to determine the effect of continuous 24-hour fumigation with hydrogen fluoride (12.4 p.p.b. v/v and 1.3 p.p.b. v/v) on the dry weight, fluoride content, and levels of free amino acids and amides, nonvolatile organic acids, free sugars, ribo-(RNA) and deoxyribonucleic (DNA) acids, and chlorophylls *a* and *b* in leaf tissues of Bonny Best tomato and Tendergreen bean plants. The high level of hydrogen fluoride used (12.4 p.p.b. v/v) was selected to induce maximum biochemical effects with a minimum amount of injury. Thus, no attempt was made to duplicate natural field conditions. During fumigation plants were harvested at 3-day intervals for 9 days. This was followed by a recovery period during which plants were again harvested at the same intervals.

In general, it was found that during fumigation at 12.4 p.p.b. v/v there was a reduced growth of tomato leaves, although this trend was reversed by the end of the recovery period. In beans there was a small but consistent reduction in yield of leaves.

The total fluoride content and the fluoride concentration of both tomato and bean leaf tissues decreased during the recovery period. This was not a dilution effect due to plant growth. The free sugar content of leaves was reduced in both species during the fumigation and recovery period. In general, nonvolatile organic acids increased after 3 days of fumigation. By 6 days the level approached that of the control and then dropped to a minimum at 9 days (or after 3 days of recovery in beans). During the post-exposure period the organic acid level recovered and, in tomatoes, again exceeded that of the control.

In bean leaves the DNA-phosphorus changed little in fumigated tissues, while it declined in the controls. RNA-phosphorus in fumigated tomato leaves was generally lower than in controls. Little difference was found between RNA-P in control or treated bean leaves. Hydrogen fluoride fumigation had a slight inhibitory effect on formation of chlorophyll *a* and *b* of leaf tissues, but no inhibition was evident by the end of the recovery period.

In a separate experiment respiration was stimulated in tomato and bean leaves by 67 and 72 per cent, respectively, by hydrogen fluoride fumigation for 8 days at 1.6 p.p.b. v/v.

The observed effects on metabolism do not constitute evidence for inhibition of enolase. Increased respiration observed in a number of plant species also is not clearly compatible with this theory. It is suggested that fluoride may exert its effect by interfering with phosphorus metabolism.

Emphasis is placed on the length of fumigation and its influence on the results obtained. For example, under the conditions of the experiment, if the fumigation period was set arbitrarily for 3 days, there would have been an increased level of organic acids. With a fumigation period of 6 days, no change would have been noted, while at 9 days it would have been concluded that fluoride reduced the organic acid level. Thus, depending upon the length of fumigation alone, positive interpretations could have been made that fluoride stimulates, has no effect upon, or decreases the organic acid level of plants.

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INTRODUCTION

There are innumerable reports which implicate fluorides as inhibitors of enzyme systems *in vitro*. Among these enzymes are phosphatases (32, 35, 44), phosphorylases (44), succinic dehydrogenase (48), glutamine synthetase (15), phosphoglucomutase (14, 36), enolase (34, 51), amylase (45), cytochrome *c* (8), polyphenoloxidase (23), peroxidase (8), and rat muscle adenosinetriphosphatase (21).

Little is known about the specific action of fluorides within the living cell, although many physiological effects have been noted. A great deal, however, is known about the mode of action of fluoroacetate, a compound known to occur in the African plant, gifblaar (*Dichapetalum cymosum* Hook.). Conversion of fluoroacetate to fluoroacetyl coenzyme A has been demonstrated (31). Condensation of fluoroacetyl coenzyme A with oxalacetate by citrogenase yields fluorocitrate, a potent inhibitor of aconitase (9, 12, 39, 40), and, as a result, citrate is accumulated. More recent investigations have shown that fluoromalate inhibits malic enzyme and oxalacetic decarboxylase of rat liver mitochondria (16), while fluoropyruvate inhibited pyruvate oxidation in guinea pig kidney, pigeon brain particles, and yeast (17).

Fluorides were reported as early as 1890 to result in a reduction in the rate of yeast fermentation (8). By 1892 Arthus and Huber (4) had reported fluoride inhibition of cellular respiration in microorganisms. It is generally assumed, however, that the glycolytic pathway is more sensitive to fluoride than is aerobic respiration (8). Reduction in plant growth and inhibition of physiological processes by fluoride have been often ascribed to effects on enolase (33, 46), which is known to be extremely sensitive to fluoride *in vitro* (34, 52).

As a result of field studies with peach and prune leaves, McNulty and Newman (29) concluded that atmospheric fluorides inhibit respiration. In a later report (30) of studies in which beans were exposed to 30 p.p.b. of hydrogen fluoride for up to 18 days and gladiolus to 2.4 p.p.b. for 25 days, respiration was increased. Stimulated respiration was assumed to be due to tissue injury. Other investigators have also found the respiratory rate to be stimulated by fluoride under varying growth or nutritional conditions (1, 2, 27). Hill *et al.* (19) reported that relatively long low-level fumigations of seven plant species resulted in no effect on respiration except where injury occurred. When injury was induced by scalding, crushing, or burning, respiration was also stimulated.

McNulty and Lords (28) have recently reported that fluoride induced a stimulation of oxygen consumption and at the same time increased the total phosphorylated nucleotides. They have suggested that fluoride disrupts the basic energetics of the cell, resulting in increased oxygen consumption due to an increased amount of phosphate acceptor or donor, or, at least, by interfering with the interrelationships between inorganic phosphorus, adenosinediphosphate, and adenosinetriphosphate.

Ross *et al.* (46) have presented evidence that fluoride sensitivity of plants is related to their respiratory pathway. They found that fluoride-sensitive varieties of gladiolus had a higher C_6/C_1 ratio than resistant varieties. Thus, they concluded that sensitive varieties metabolize primarily via the Embden-Meyerhof pathway, while resistant varieties are more dependent upon the pentose phosphate pathway. This difference, however, was not consistent for other plant species, and they concluded that species differences in fluoride sensitivity are not necessarily related to the respiratory pathway. Infiltration of leaves of *Polygonum orientale* with potassium fluoride resulted in increased respiration and decreased C_6/C_1 ratio. This was interpreted to mean that a fluoride-induced inhibition of enolase causes a shift in the respiratory mechanism to the less sensitive pentose phosphate pathway.

The effect of fluorides on the chlorophyll content of leaves has been studied by McNulty and Newman (29, 37), who found reduced levels of chlorophyll *a*, chlorophyll *b*, and protochlorophyll. Effects on other biochemical constituents have not been extensively studied. Bonner and Thimann (7), studying the effect of sodium fluoride on *Avena* coleoptiles and pea stem sections, found that growth was inhibited. The response was not ascribed to inhibition of enolase. Other workers have shown that the growth inhibition by sodium fluoride of excised plant parts resulted in a lower level of sugars and cell wall carbohydrates (13). Hydrogen fluoride supplied at a rate of 25 p.p.m. to citrus trees grown in nutrient culture was shown to increase the citric and ascorbic acid content of fruits and to reduce growth and yield (11). To what extent root injury from hydrogen fluoride was involved was not made clear.

The experiments reported herein present data from a time-course study on the effects of a relatively high level and a relatively low level of atmospheric hydrogen fluoride on some constituents of leaves of Bonny Best tomato and Tendergreen bean plants.

MATERIALS AND METHODS

Eighty-four individually potted plants of Bonny Best tomato (*Lycopersicon esculentum* Mill.), 5 weeks old, and 84 pots of Tendergreen bean (*Phaseolus vulgaris* L.), 12 days old and containing 6 plants per pot, were selected for uniformity. Forty-two pots of each were randomly distributed in control and fumigation chambers.

Plants in the control chamber were treated with water-scrubbed air (containing less than 0.2 p.p.b. of hydrogen fluoride v/v), and those in the fumigation chamber were subjected during exposure to an average concentration of hydrogen fluoride of 12.4 p.p.b. v/v. The fumigations were on a continuous 24-hour basis. The rate of air flow in both chambers was the same. The relatively high concentration of hydrogen fluoride was chosen in order to insure that some biochemical changes would be induced, while not producing severe injury of the plants. At periods of 3, 6, and 9 days after the experiment was initiated, 7 pots each of tomatoes and beans (7 tomato and 42 bean plants) were removed from the chambers by random selection, and all leaf tissues were harvested as quickly as possible. The remaining plants (for recovery studies) were removed from the chambers and realigned randomly in the greenhouse. After a recovery period of 3, 6, or 9 days, leaf tissues from 7 pots of plants were harvested by random selection. All plants were watered from saucers to prevent possible removal of fluorides from the leaves due to washing. This is in keeping with the report of Ledbetter *et al.* (25) that 47 per cent of the accumulated fluoride could be removed from tomato leaves by three dips in distilled water. Thus, harvests were made over a 9-day fumigation and a 9-day recovery period. No symptoms of injury were evident on treated plants throughout the experiment. The experiment was carried out during the period from December 2 to 19, 1958.

Harvested leaf tissues were weighed, cut into small pieces, and aliquots were dried at 72° C. in a forced-draft oven for dry weight and fluoride determinations. The remaining fresh tissue was extracted five times with 80 per cent ethanol. The combined ethanolic extracts were treated with hydrogen-form Dowex 50-X8 and acetate-form Dowex 1-X10 to take up amino compounds and nonvolatile organic acids, respectively, and sugars were extracted from the filtrate after resin treatment (53). Alcohol-insoluble residues were lyophilized.

Free amino acids and amides were eluted from Dowex 50 by the method of Plaisted (41) and were analyzed quantitatively by the two-dimensional paper chromatographic procedure of Porter *et al.* (43). Nonvolatile organic acids were separated on Dowex 1 by gradient elution with $2.5N$ acetic acid followed by $6N$ formic acid (38, 54). Individual acids were further identified by one-dimensional paper chromatography. Sugars were separated and analyzed as reported earlier (53), except that descending one-dimensional chromatography on Whatman No. 1 paper was employed. Chlorophylls *a* and *b* were determined by the method of Arnon (3). Fluoride was determined by the method of Gwirtsman *et al.* (18). Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were determined by differential perchloric acid extraction of alcohol-insoluble residues (20), followed by total phosphorus analyses (42).

A portion of this experiment was repeated at a later date (September 22 to October 9, 1959) with plants exposed to hydrogen fluoride at an average concentration of 1.3 p.p.b. v/v. Both leaf and stem tissues were analyzed for fluoride.

Oxygen uptake of leaf punches (22) was measured in a third experiment run from April 8 to 15, 1959, in which bean and tomato plants were exposed to 1.6 p.p.b. v/v of hydrogen fluoride for 8 days. Twelve 19-mm. punches of the youngest fully expanded leaves were used in each Warburg vessel. All analytical determinations were at least duplicated.

RESULTS

Dry weights of bean and tomato leaf tissues are shown in Figure 1. In tomatoes, fumigation had a depressing effect on dry weight yields, although this trend was reversed by the end of the recovery period. There was a small but consistent reduced yield of bean leaves.

As might be expected, the fluoride content (p.p.m.) of leaf tissues increased as the fumigation period was extended, but the concentration decreased following removal of the plants from the fumigation chambers (Fig. 2). Since the decreased concentration of fluoride in the tissues corresponds to a period of rapid plant growth, decreased fluoride levels could be attributed to a dilution of existing fluoride. However, when the data are expressed on the basis of micrograms per plant (Fig. 2), the results strongly suggest a loss of fluoride from leaf tissues during the post-exposure period. When this portion of the experiment was repeated with a lower level of

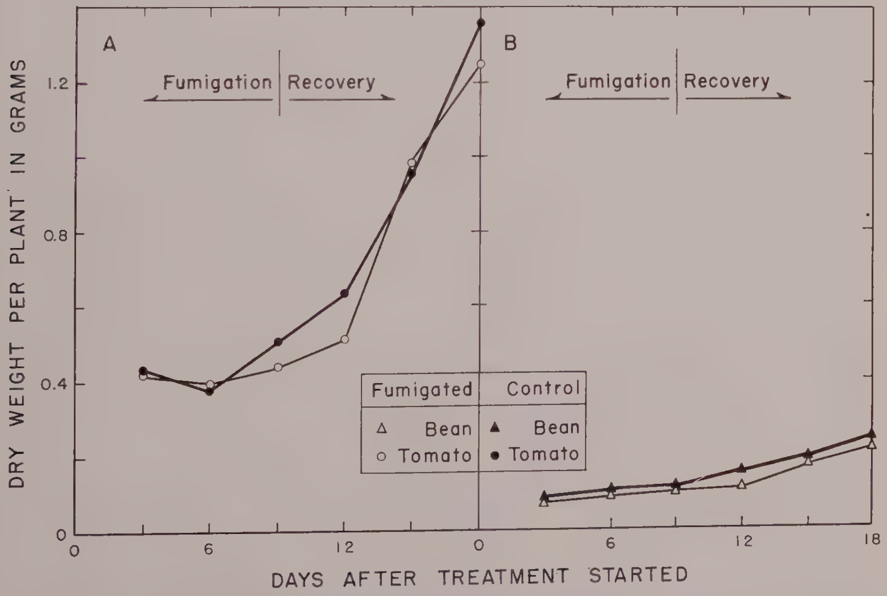

FIGURE 1. Effect of a 9-day hydrogen fluoride fumigation (12.4 p.p.b. v/v) and a 9-day recovery period on the dry weight yield of leaf tissues of (A) Bonny Best tomato, and (B) Tendergreen bean plants.

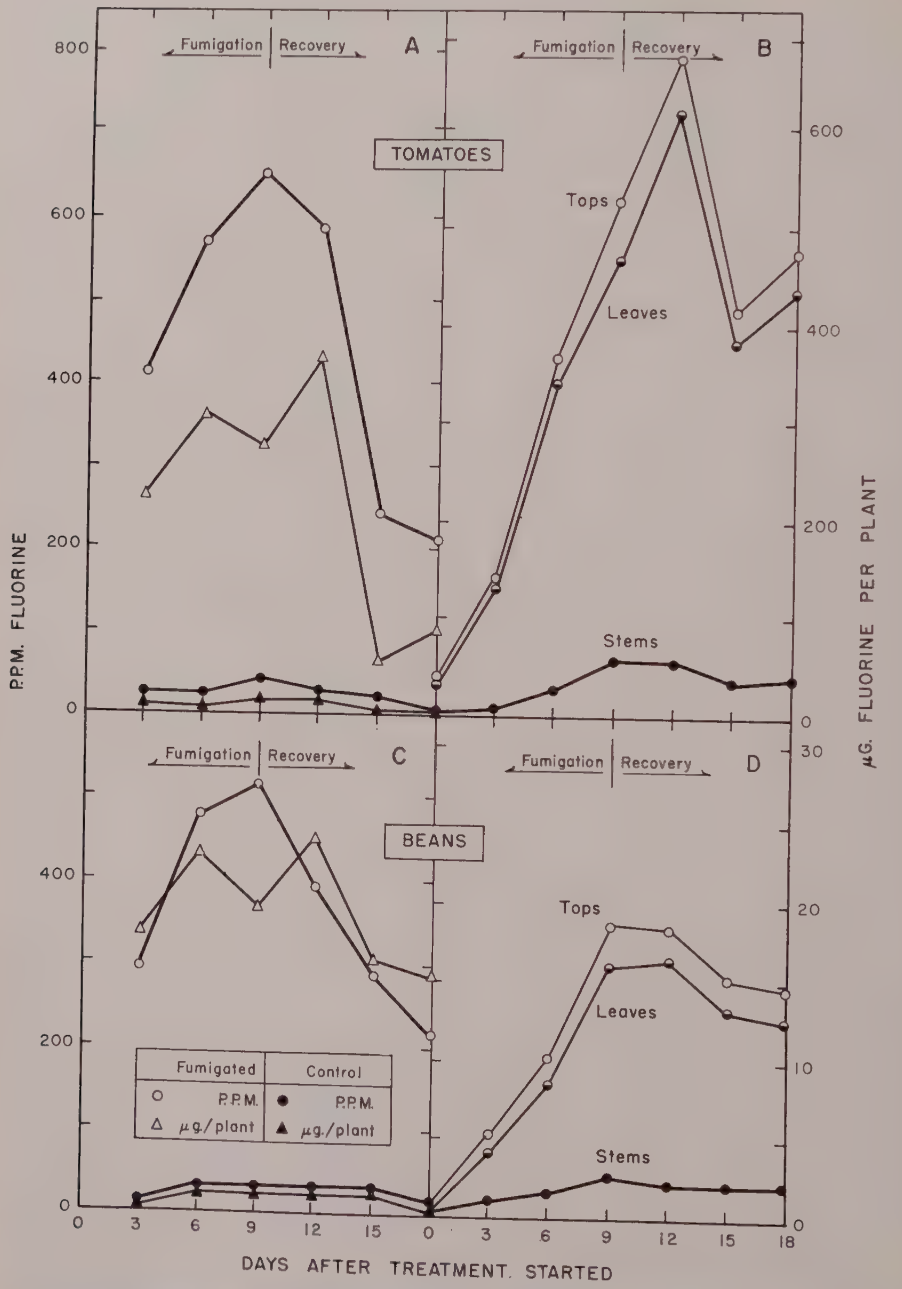

FIGURE 2. Effect of a 9-day hydrogen fluoride fumigation and a 9-day recovery period on the fluoride content of leaf tissues of Bonny Best tomato plants exposed to (A) 12.4 p.p.b. v/v, and (B) 1.3 p.p.b. v/v, and of leaf tissues of Tendergreen bean plants exposed to (C) 12.4 p.p.b. v/v, and (D) 1.3 p.p.b. v/v.

hydrogen fluoride, there was a clear indication of a loss of fluoride from leaf and stem tissues of both beans and tomatoes during the post-exposure period (Fig. 2). It may be pertinent to note that in the second experiment, which was carried out in early fall and employed a level of fluoride about one-tenth of that used during the December experiment, the fluoride content of tomato leaves was nearly twice as much as in the earlier experiment. In beans there was about half as much fluoride as in the December experiment.

Total free amino acid and amide data are presented in Table I. Changes in individual amino acids and amides are not presented since there were no significant or consistent differences recorded. Results for total amino acids and amides, similarly, were not sufficiently different to warrant further discussion beyond the fact that some differences in these constituents do occur.

During the first 6 days of fumigation no change in the individual or total free sugars of tomato leaves is apparent (Table II). Data for the 9-day fumigation harvest are not available. After 3 days of recovery, however, the sugar level of fumigated tissues was lower than that of the control, and this difference persisted through the recovery harvests. The total content of sugars in both control and fumigated tissues increased throughout the 18-day experiment. In bean leaves individual and total

TABLE I

EFFECT OF HYDROGEN FLUORIDE FUMIGATION (12.4 P.P.B. V/V) AND A RECOVERY PERIOD ON THE FREE AMINO ACID AND AMIDE CONTENT OF BONNY BEST TOMATO AND TENDERGREEN BEAN LEAVES

Time after initiation of fumigation period (days)	Quantity in milligrams per plant					
	Amino acids and amides		Amino nitrogen		Amide nitrogen	
	Control	Fumigated	Control	Fumigated	Control	Fumigated
			Tomato			
3 (Fumigation)	7.51	8.52	1.00	1.16	0.16	0.21
6 “	8.51	7.67	1.12	0.98	0.33	0.32
9 “	4.01	5.10	0.45	0.57	0.15	0.23
12 (Recovery)	3.20	4.26	0.39	0.50	0.07	0.09
15 “	3.95	3.64	0.47	0.42	0.07	0.07
18 “	3.38	3.61	0.40	0.43	0.03	0.05
			Bean			
3 (Fumigation)	1.27	1.26	0.16	0.15	0.06	0.07
6 “	1.43	1.11	0.18	0.15	0.08	0.06
9 “	0.82	1.42	0.09	0.19	0.04	0.09
12 (Recovery)	0.71	0.77	0.08	0.09	0.02	0.04
15 “	0.80	0.87	0.09	0.10	0.01	0.03
18 “	0.98	0.83	0.12	0.10	0.01	0.01

TABLE II

EFFECT OF HYDROGEN FLUORIDE FUMIGATION (12.4 P.P.B. V/V) AND A RECOVERY PERIOD ON THE SOLUBLE SUGAR CONTENT OF BONNY BEST TOMATO AND TENDERGREEN BEAN LEAVES

Time after initiation of fumigation period (days)	Quantity in milligrams per plant							
	Fructose		Glucose		Sucrose		Total	
	Control	Fumigated	Control	Fumigated	Control	Fumigated	Control	Fumigated
	Tomato							
3 (Fumigation)	0.25	0.24	0.42	0.37	0.44	0.40	1.11	1.01
6 “	0.63	0.58	0.67	0.75	0.37	0.37	1.67	1.70
9 “	0.61	—	0.76	—	0.82	—	2.19	—
12 (Recovery)	2.28	1.75	2.54	1.27	1.17	0.63	5.99	3.65
15 “	2.81	2.35	2.78	2.47	1.94	1.48	7.53	6.30
18 “	3.03	2.90	3.15	2.29	1.84	1.11	8.02	6.30
	Bean							
3 (Fumigation)	0.16	0.04	0.17	0.09	0.26	0.16	0.59	0.29
6 “	0.08	0.06	0.15	0.08	0.23	0.09	0.46	0.23
9 “	0.28	0.11	0.25	0.19	0.96	0.55	1.49	0.85
12 (Recovery)	1.00	0.12	1.35	0.18	1.30	0.42	3.65	0.72
15 “	0.84	0.45	0.98	0.92	1.37	0.63	3.19	2.00
18 “	1.28	1.11	2.65	1.93	1.34	0.83	5.27	3.87

sugars decreased after 3 days of fumigation. This effect persisted throughout the fumigation and recovery periods.

Fumigation of tomato plants for 3 days resulted in an increase in malic acid over the controls (Table III and Fig. 3). By 6 days the level had dropped to nearly that of the control. It then dropped to its lowest comparative level by the 9-day harvest. After removal from the fumigation chambers for 6 days, the amount of acid approached that of the controls. This was followed by another period of stimulation. Citric acid followed a similar pattern. In beans there was an initial stimulation, followed by decreasing amounts of malic acid, reaching a minimum concentration at the 3-day recovery harvest. The malic acid then approached that of the control at the subsequent 6- and 9-day recovery harvests. The concentration of citric-malonic acids followed a pattern similar to that for malic acid. Both species, then, exhibited an early stimulatory period, followed by a period of inhibition closely associated with the end of the fumigation period. During the post-fumigation period there was a recovery in the amount of acid in both plants, and, in the case of tomatoes, a second period of stimulation.

DNA-phosphorus of control and fumigated tomato leaves increased during the entire course of the experiment (Table IV), with more in the

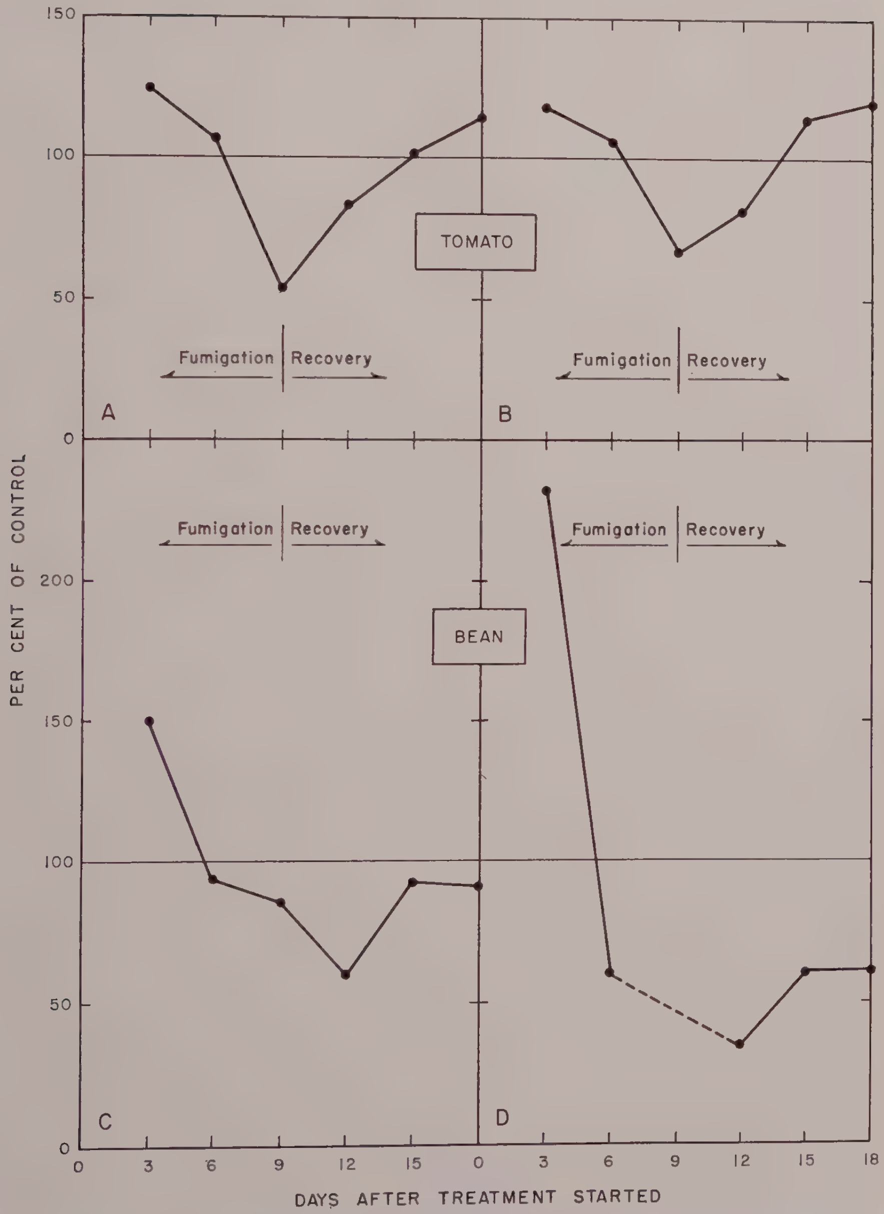

FIGURE 3. Effect of a 9-day hydrogen fluoride fumigation (12.4 p.p.b. v/v) and a 9-day recovery period on (A) malic and (B) citric acid content of Bonny Best tomato leaves, and on the (C) malic and (D) citric-malonic acid content of Tendergreen bean leaves.

TABLE III

EFFECT OF HYDROGEN FLUORIDE FUMIGATION (12.4 P.P.B. V/V) AND A RECOVERY PERIOD ON THE MAJOR NONVOLATILE ORGANIC ACIDS OF BONNY BEST TOMATO AND TENDERGREEN BEAN LEAVES

Time after Initiation of fumigation period (days)	Quantity in milligrams per plant					
	Malic		Citric		Total	
	Control	Fumigated	Control	Fumigated	Control	Fumigated
			Tomato			
3 (Fumigation)	7.1	8.8	22.3	26.3	29.4	35.1
6 “	5.8	6.1	14.0	14.9	19.8	21.0
9 “	20.4	10.9	29.4	29.4	49.8	40.3
12 (Recovery)	68.5	56.9	43.1	35.3	111.6	92.2
15 “	120.0	118.7	75.1	86.9	195.1	205.6
18 “	124.2	142.0	83.4	99.8	207.6	241.8

Time after initiation of fumigation period (days)	Quantity in milligrams per plant							
	Quinic, glyceric, unknown		Malic		Citric, malonic*		Total	
	Control	Fumigated	Control	Fumigated	Control	Fumigated	Control	Fumigated
				Bean				
3 (Fumigation)	2.1	2.0	1.2	1.8	1.5	3.9	4.8	7.7
6 “	2.4	2.7	3.0	2.8	3.5	2.4	8.9	7.9
9 “	3.6	3.9	6.8	5.8	—	—	—	—
12 (Recovery)	6.2	4.9	19.9	11.9	9.2	3.1	35.3	19.9
15 “	9.9	7.5	32.1	29.5	28.5	17.2	70.5	54.2
18 “	9.4	7.4	36.9	33.4	46.3	28.3	92.6	69.1

* Major constituent in fraction.

control leaves. Differences between treatments, however, showed up only during the recovery period. In beans DNA-phosphorus declined in control tissues, while there was little change in fumigated tissues. RNA-phosphorus of control tomato leaves increased until the first recovery harvest, then declined. In fumigated leaves the maximum concentration was also attained at this harvest, but the levels were generally lower than those of controls both during and, especially, after fumigation. In control and fumigated beans, RNA-phosphorus declined for the first three harvests, increased by the first recovery harvest, then declined again, with no apparent differences in the amount in control and fumigated tissues.

Fumigation by hydrogen fluoride appeared to have a slight effect on the chlorophyll *a* and *b* content of leaf tissues (Table V), but the differences are by no means striking. If differences noted are real, they are certainly not evident by the final post-exposure harvest.

TABLE IV

EFFECT OF HYDROGEN FLUORIDE FUMIGATION (12.4 P.P.B. V/V) AND A RECOVERY PERIOD ON THE RNA- AND DNA-PHOSPHORUS CONTENTS OF BONNY BEST TOMATO AND TENDERGREEN BEAN LEAVES

Time after initiation of fumigation period (days)	Quantity in milligrams per plant			
	DNA-P		RNA-P	
	Control	Fumigated	Control	Fumigated
	Tomato			
3 (Fumigation)	0.093	0.093	0.593	0.559
6 “	0.094	0.090	0.600	0.574
9 “	0.100	0.092	0.607	0.566
12 (Recovery)	0.126	0.096	0.976	0.740
15 “	0.116	0.108	0.739	0.586
18 “	0.140	0.106	0.668	0.598
	Bean			
3 (Fumigation)	0.019	0.018	0.126	0.127
6 “	0.015	0.015	0.093	0.104
9 “	0.016	0.016	0.081	0.068
12 (Recovery)	0.015	0.018	0.141	0.134
15 “	0.015	0.018	0.116	0.108
18 “	0.012	—	0.090	—

TABLE V

EFFECT OF HYDROGEN FLUORIDE FUMIGATION (12.4 P.P.B. V/V) AND A RECOVERY PERIOD ON THE CHLOROPHYLL A AND CHLOROPHYLL B CONTENTS OF BONNY BEST TOMATO AND TENDERGREEN BEAN LEAVES

Time after initiation of fumigation period (days)	Quantity in milligrams per plant					
	Chlorophyll a		Chlorophyll b		Total	
	Control	Fumigated	Control	Fumigated	Control	Fumigated
	Tomato					
3 (Fumigation)	4.29	4.06	1.82	1.79	6.11	5.85
6 “	4.00	4.13	1.70	1.82	5.70	5.95
9 “	4.84	4.45	2.37	2.03	7.21	6.48
12 (Recovery)	5.23	4.74	2.16	2.06	7.39	6.80
15 “	—	—	—	—	—	—
18 “	8.56	8.83	3.73	3.84	12.29	12.67
	Bean					
3 (Fumigation)	0.83	0.81	0.36	0.36	1.19	1.17
6 “	0.80	0.67	0.39	0.32	1.19	0.99
9 “	0.90	0.87	0.44	0.44	1.34	1.31
12 (Recovery)	1.10	0.99	0.48	0.44	1.58	1.43
15 “	—	—	—	—	—	—
18 “	1.74	1.73	0.72	0.86	2.46	2.59

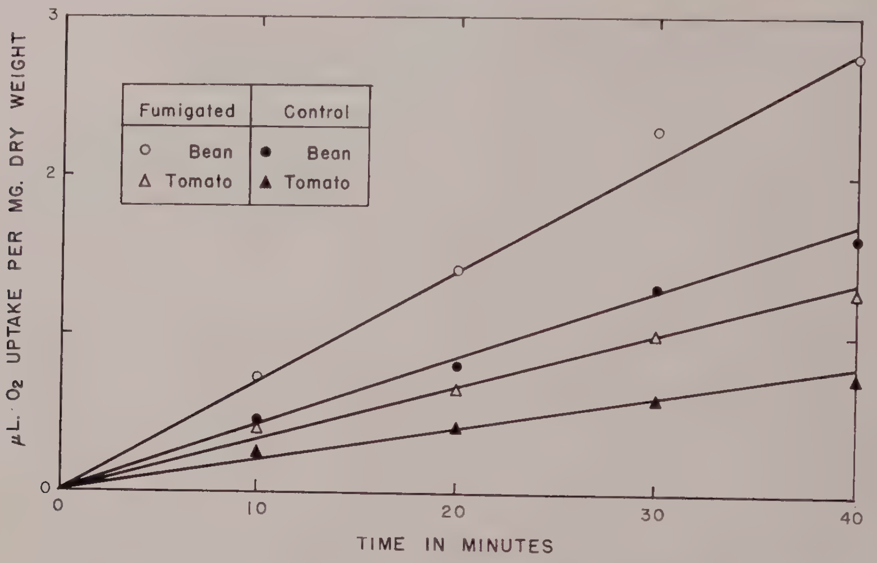

FIGURE 4. Effect of an 8-day hydrogen fluoride fumigation (1.6 p.p.b. v/v) on the O_2 uptake of tissues of Bonny Best tomato and Tendergreen bean leaves.

Fumigation of tomato and beans with hydrogen fluoride at 1.6 p.p.b. for 8 days stimulated respiration by 67 and 72 per cent, respectively (Fig. 4).

DISCUSSION

It has not been the purpose of the present investigation to study and duplicate the effects of atmospheric fluorides under realistic field conditions. First of all, the level of hydrogen fluoride used for the major portion of these experiments (12.4 p.p.b. v/v) was too high to approximate any but the most unusual atmospheric pollution conditions. Secondly, these experiments were carried out with greenhouse-grown plants fumigated under greenhouse conditions; thus, the softer, more succulent growth may have been more predisposed than field-grown plants to biochemical changes due to fluorides. Lastly, the fumigations were carried out on a continuous basis for 3, 6, or 9 days at a relatively constant level of hydrogen fluoride, a condition of uniformity not usually associated with field conditions. These experiments, then, were designed in an attempt to induce maximum biochemical effects without producing severe injury to the plants.

The apparent loss of fluoride from fumigated plants (Fig. 2) has been reported by others. This includes the loss of fluorides from plants which had absorbed them from soil and from nutrient solutions (50, 55). The evidence apparently is not indisputable; other workers (10) have reported that fluoride absorbed by tomato roots was not lost, although fluoride from hydrogen fluoride fumigation was lost during a recovery period. In the present study losses of fluoride from plants are based on results for leaves or for leaves and stems. However, since results of other workers (10, 11, 25) have shown that translocation of fluoride from tops to roots of plants is relatively small, it is felt that the loss of fluoride reported is due to loss from leaves and not to root excretion.

It has been a common practice in recent years to ascribe most growth and physiological effects of fluoride on plants to inhibition of enolase. Whether or not this is true remains to be established experimentally. Conventional assays for enolase in plant tissues suffer from the possibility of a fluoride inhibition occurring during the maceration and extraction procedure and may not reflect *in vivo* conditions. Miller (33) has shown that hydrogen fluoride-fumigated plants sprayed with pyruvate exhibited restored enolase activity. Normal growth was only partially restored to pyruvate-sprayed plants, however, suggesting that reduced growth was not due only to reduced enolase activity.

The effect of fluoride on the respiration of tissue homogenates, detached parts of plants, or intact plants has never been clearly established. Both reduced respiratory activity (5, 6, 13, 24, 29) and increased respiratory activity (2, 27, 30, 46, 49) have been reported. Some investigators feel that increased respiration is due not merely to the presence of tissue fluoride, but rather to physical injury caused by high concentrations of fluoride (19, 30). This is contradicted by reports of increased respiratory activity with no visible tissue injury (2, 27). Other studies[1] have shown that fumigation for 7 to 9 days at from 1 to 3 p.p.b. v/v of hydrogen fluoride stimulated respiration of leaves of Martin's milo maize, Bonny Best tomato, Tendergreen bean, and Snow Princess and Elizabeth the Queen gladiolus. Maize exhibited symptoms of marginal and intercostal chlorosis, while the gladiolus varieties showed tip necrosis. Other species exhibited no fluoride injury symptoms. Respiration of leaves of Triumph sweet potato and of wheat seedlings was unaffected, while that of Jerusalem artichoke was lowered. No visible symptoms were evident on these plants. The respiratory quotient of all plants was unaffected, except that of Jerusalem artichoke which was lower. It is quite clear that the fluoride effect on tissue respiration is intimately associated with the concentration of fluoride that reaches active cellular sites, as modified by conditions of light, temperature, time, and other factors. The fact remains, nevertheless, that increased respiration is not compatible with enolase inhibition, unless the respiratory mechanism is altered to such an extent that this enzyme is bypassed. Evidence that potassium fluoride infiltration shifts the respiratory pathway from the Embden-Meyerhof and Krebs cycle to the pentose phosphate pathway in leaves of *Polygonum orientale* has been presented (46). It is not clear why the respiratory rate, however, should necessarily be higher.

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[1] Weinstein, Leonard H., unpublished.

None of the data reported herein point to the ability of plants to form fluoroacetate as a result of hydrogen fluoride fumigation since no marked accumulation of citrate, even at the 3-day harvest, was found. The data do suggest another possible mechanism of action, involving the uncoupling of phosphorylation or reduced transphosphorylating ability by fluorides or by a product of fluoride metabolism. The competitive inhibition of a phosphorylase by fluorophosphate has been reported (44). Fluorophosphate could be formed by "fluorokinase" which, in rabbit muscle, has been shown to be identical to pyruvic kinase (51). The reaction, ATP+fluoride CO_2, $Mg^{++} \rightarrow$ADP+fluorophosphate, results in 1) the formation of an enzyme inhibitor, and 2) the loss of a high energy bond.

If, indeed, fluoride exerts its effect by reduced phosphorylative activity, there would be decreased dependence of respiration on this system similar to that ascribed for 2,4-dinitrophenol (26, 47). This could, in turn, result in a reduced level of sugars, Krebs cycle acids, and free amino acids, similar to the results presented here. The results of McNulty and Lords (28) for sodium fluoride-treated *Chlorella*, however, suggest increased phosphorylative activity, at least into nucleotides.

The data of possibly the most significance are those for nonvolatile organic acids (Table III and Fig. 5). The great importance of length of fumigation is clearly shown. For example, if the length of fumigation had been arbitrarily set for 3 days at 12.4 p.p.b. v/v of hydrogen fluoride, other conditions being equal, it might have been concluded that hydrogen fluoride induced an increased level of organic acids in tomato leaves. With a fumigation period of 6 days the results would have shown that fluorides have little or no effect on organic acid metabolism. At 9 days, of course, the results do show a reduced organic acid level. Thus, depending upon the length of fumigation alone, logical interpretation could be made that fluorides stimulate, have no effect upon, or decrease the nonvolatile organic acids of tomato leaves.

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CYTOCHEMICAL STUDIES OF THE SHOOT APICES OF NORMAL AND PHYSIOLOGICALLY DWARFED PEACH SEEDLINGS. I. CELL WALL COMPONENTS

Florence Flemion and Carol Topping

SUMMARY

The development of the primary cell wall and subsequent deposition of wall substances in the shoot apices of peach [*Prunus persica* (L.) Batsch] were studied cytochemically. During sequential extraction of the pectic substances, hemicellulose, and the noncellulosic polysaccharides, no shrinkage of the cell diameter occurred until removal of the latter substances when only cellulose remained. These various components were present in all of the tissues examined; however, the amounts differed, depending upon whether the apices were growing rapidly, approaching arrested development, or in a dormant condition.

In the early stages of growth, whether the seedling was normal or would subsequently become physiologically dwarfed, hemicellulose was relatively high while the other components were quite low. When the cells became more mature the amount of noncellulosic polysaccharides increased. The thickened pith and cortical cell walls of the dormant shoots—whether normal or dwarfed—contained a great deal of cellulose, while the amounts of pectic substances and noncellulosic polysaccharides were considerable. On the other hand, the cellulose content was always low in the provascular and cambium tissues of the various types of seedlings at different stages of development, as well as in the apical initials of the germinating embryo and of the actively-growing shoot. The deposition of these substances prior to and during cessation of stem elongation in the physiological dwarfs appeared to be quite similar to the changes which occur during the onset of and subsequent dormancy in normal seedlings.

INTRODUCTION

The seeds of peach [*Prunus persica* (L.) Batsch], *Rhodotypos kerrioides* (Sieb. & Zucc.), and other rosaceous species, when forced to germinate without prechilling, produce physiologically dwarfed seedlings which lack the ability to elongate (1). Recent morphological studies by Holmsen (5) and Ledbetter (9) indicate that in these telescoped internodes there is a reduction in cell division and also suppression of cell elongation. In addition, the cell walls of the pith appear to be thicker and take up more stain than those of the normal plants (9), but the stage of development at which this thickening occurs was not ascertained.

In the present studies, the cell wall composition of the various tissues in the apical shoots of the actively growing, as well as of dormant, seedlings of both the normal and physiologically dwarfed peach and of the germinating embryo, was determined by the use of a cytochemical method developed by Jensen (7). After differential extraction and subsequent staining, an estimate was thereby obtained of the amounts of such various wall

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components as pectic materials, hemicellulose, noncellulosic polysaccharides, and cellulose. Some of these results have been reported in abstract form (3) while the complete data are presented in detail below.

MATERIALS AND METHODS

Peach seeds of a propagated wild strain known as "Superior Peach Pits" grown in Tennessee were purchased and stored dry in closed containers at 0.5° C. Upon removal of the hard outer coat (pericarp) the seeds with inner coats intact were mixed in moist granulated peat moss and held at 5° C. for approximately 8 to 10 weeks. Normal plants were subsequently obtained when the chilled seeds were planted in the greenhouse (2). Physiologically dwarfed seedlings were produced by placing excised embryos from unchilled seeds on moist filter paper in Petri dishes at room temperature and transferring them to soil in the greenhouse after 7 to 10 days when adequate root and shoot development had occurred.

The shoot tips (4 to 6 mm. in length) of both normal and physiologically dwarfed seedlings in various stages of development (actively growing, semidormant, and dormant), and the embryos from both chilled and unchilled seeds were rapidly frozen in isopentane held at $-160°$ C. with liquid nitrogen (8), dehydrated at $-32°$ C. ($\pm 2°$) during a 10-day period with frequent changes of methanol, infiltrated and embedded in paraffin (Fisher's Tissuemat 54° to 56° C.) and then sectioned at 10 microns (μ).[1]

The sections were mounted on slides with Haupt's adhesive and spread with 3 per cent formalin. Consecutive sections of a given tissue were divided among four slides as described and illustrated by Jensen (7, p. 288). That is, sections 1, 5, etc. were placed on slide A; 2, 6, etc. on B; 3, 7, etc. on C; and 4, 8, etc. on D, then dewaxed and parlodion coated. Slide A served as control while slides B, C, and D were treated with 0.5 per cent ammonium oxalate for removal of the pectic substances. Slide B was set aside (minus pectin) and C and D were treated with 4 per cent sodium hydroxide solution for extraction of the hemicellulose. Slide C was set aside (minus pectin and hemicellulose) and slide D was then treated with 17.5 per cent sodium hydroxide for extraction of the insoluble noncellulosic polysaccharides, leaving only cellulose. After thorough drying, the sodium hydroxide crystals were removed from slides C and D with ethanol (7, p. 289) and the sections on slides B, C, and D were again parlodion coated.

The extraction of these various cell wall constituents has been fully described by Jensen (7) and the technique was followed in detail except for the following changes. The temperature during the extractions for the pectic substances was 75° C. and for the hemicellulose and noncellulosic polysaccharides approximately 22° C. The extraction period was 16 hours, during which the slides resting on small pieces of glass in large Petri dishes were completely submerged. These dishes were not moved until the solvents had been withdrawn. The ammonium oxalate was removed with very gentle suction through a narrow necked pipette (7), but when this method was attempted with either of the sodium hydroxide solutions there was a loss of material due to the movement of the loosened sections. By reducing surface tension (solvents in 5 per cent ethanol) and removing the solution very slowly with glass wicks practically all of the sections remained in position on the slides. To prevent loss of sections during the removal of the NaOH crystals and subsequent coating with parlodion, the slides were maintained in a horizontal position.

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[1] The technical assistance of Miss Margaret A. Masters in the preliminary phases of these experiments is gratefully acknowledged.

For staining the cell walls in the control (A) and extracted (B, C, and D) tissues, periodic acid Schiff's reagent for polysaccharides was used (7, p. 289). The intensity of color obtained, prior to and following each extraction, served to indicate the amount and localization of the various wall components. Since methanol, various ethanolic solutions, as well as water, were used during fixation, hydration, etc., only the materials insoluble in these liquids were detectable by the cytochemical technique employed. In order to maintain uniformity, the four slides of a given set were always taken through the staining procedure together and, in addition, the various series which were to be compared (such as those in Fig. 1) were all stained with the same reagents on a given day.

Black and white photomicrographs of the stained sections were taken and developed under rigidly controlled conditions by the Institute's illustration division under the direction of William G. Smith, Jr. The prints were compared with the original material and, although in some instances the differences in color intensity were greater than they appeared to be in the photographs, the gradient in a given series was always visible in the prints.

RESULTS

The shoot apices of normal seedlings, shown in Figure 1, illustrate that the various wall components—pectic materials, hemicellulose, noncellulosic polysaccharides and cellulose—differed quantitatively, depending upon whether the seedlings were actively growing (top row), approaching arrested development after 10 weeks (center row), or in deep dormancy after 20 weeks (bottom row). The extremely pale, thin walls of the young, rapidly-growing apex of a seedling (approximate height of 78 mm., 3 weeks after planting adequately chilled seed in the greenhouse) shown in the top row on the extreme right indicate that the cellulose content was quite low, while the somewhat darker walls in the adjacent photograph (second from right) reveal the presence of some noncellulosic polysaccharides. The hemicellulose content was relatively high since the walls stained quite deeply

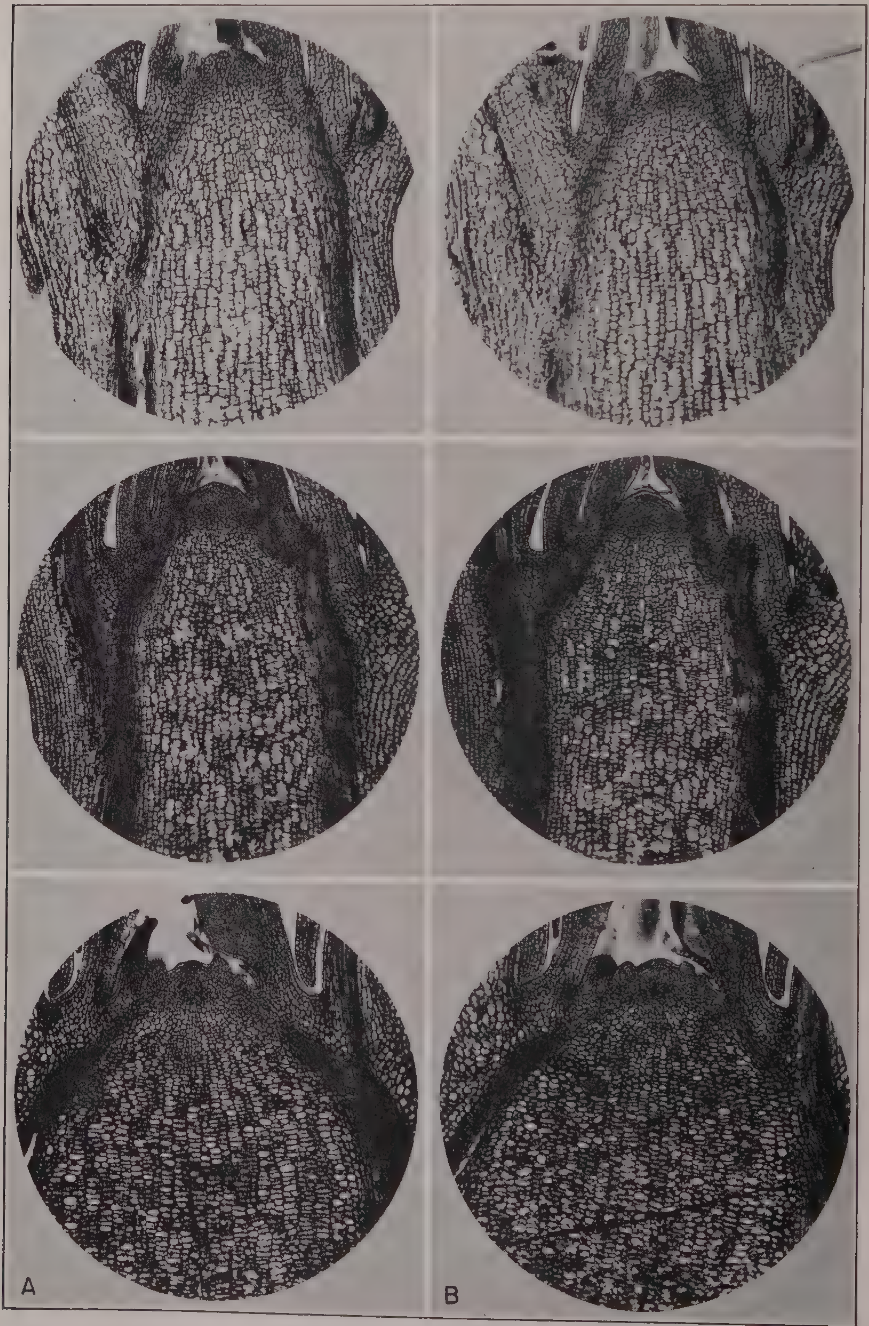
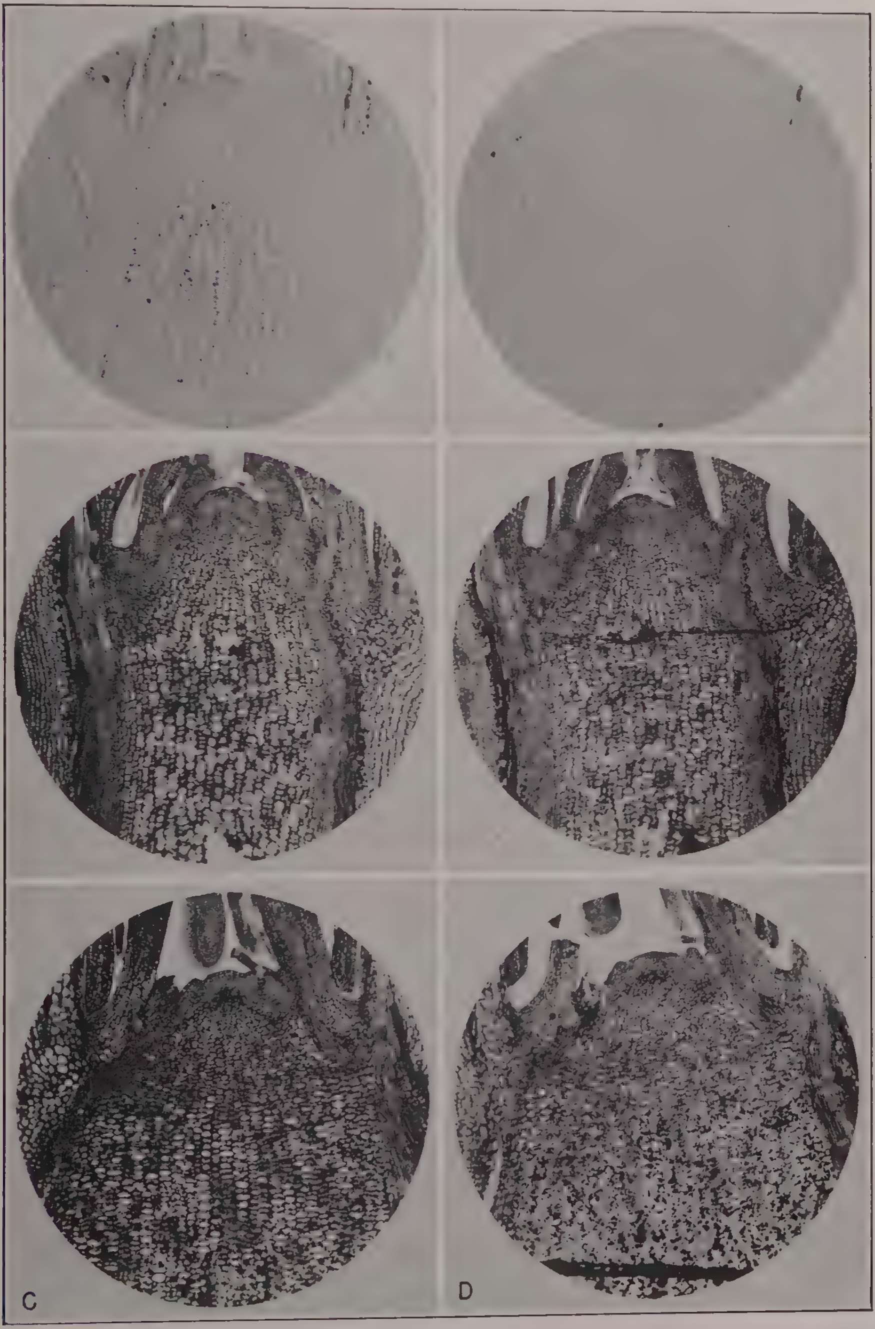


FIGURE 1. Shoot apices from normal peach seedlings grown from chilled seeds in the greenhouse for: 3 (top row), 10 (center row), and 20 (bottom row) weeks. Left to right: successive sections (10 microns each) taken near median and stained after differential extrac-



tion: (A) Controls containing all components; (B) pectic substances extracted; (C) pectic substances and hemicellulose extracted; and (D) pectic substances, hemicellulose and non-cellulosic polysaccharides extracted. (×58.75)

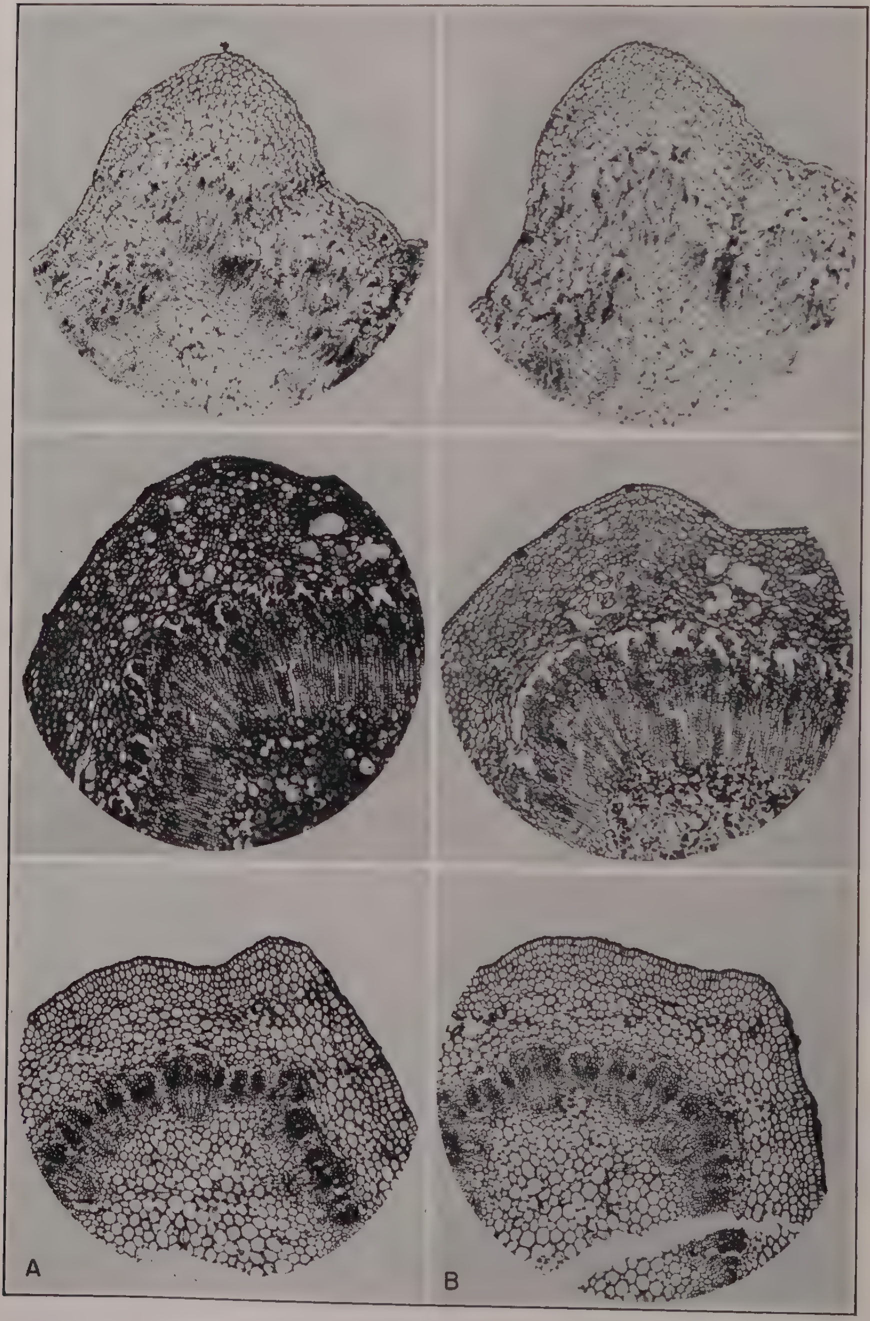
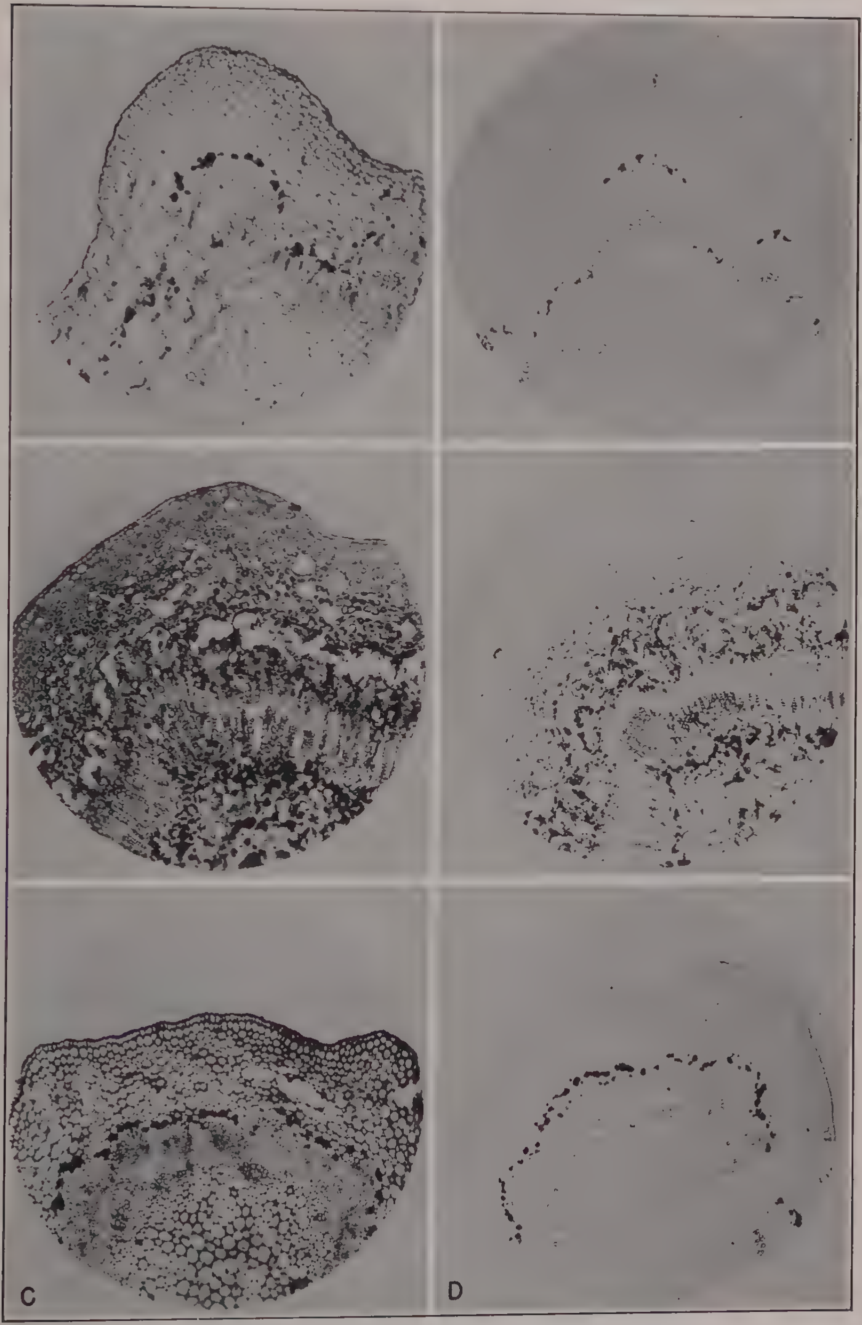


FIGURE 2. Cross sections of shoot apices of normal and dwarf peach seedlings grown in greenhouse. Sequential sections: top row made 6,500 μ from tip of rapidly growing normal plant 3 weeks after seed was sown; middle row made 1,000 μ from tip of dormant normal



seedlings 20 weeks after seed was sown; and bottom row made 3,350 μ from tip of actively growing physiological dwarf. Treatment—A, B, C, D are as described for Figure 1. (×58.75)

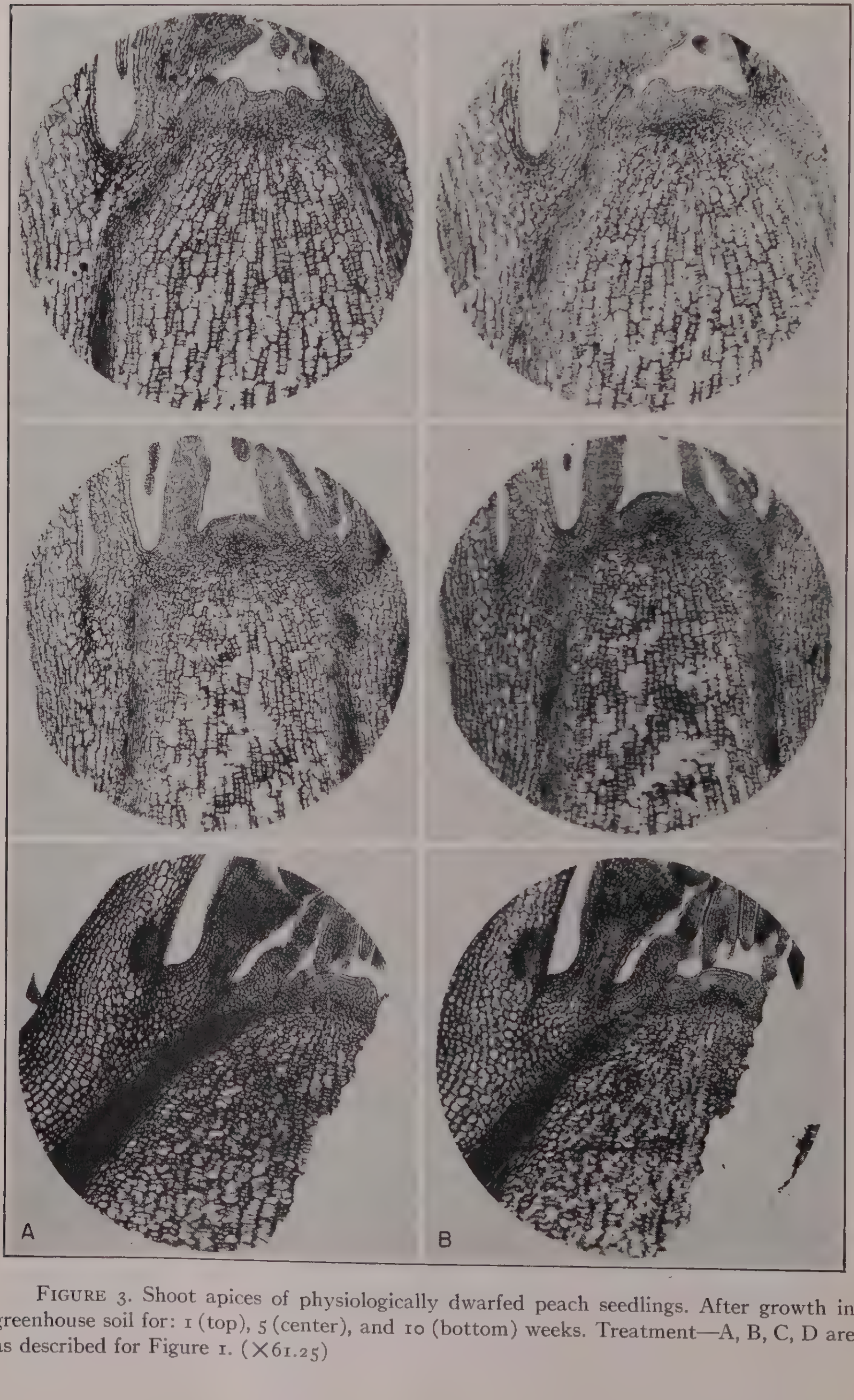


FIGURE 3. Shoot apices of physiologically dwarfed peach seedlings. After growth in greenhouse soil for: 1 (top), 5 (center), and 10 (bottom) weeks. Treatment—A, B, C, D are as described for Figure 1. (×61.25)

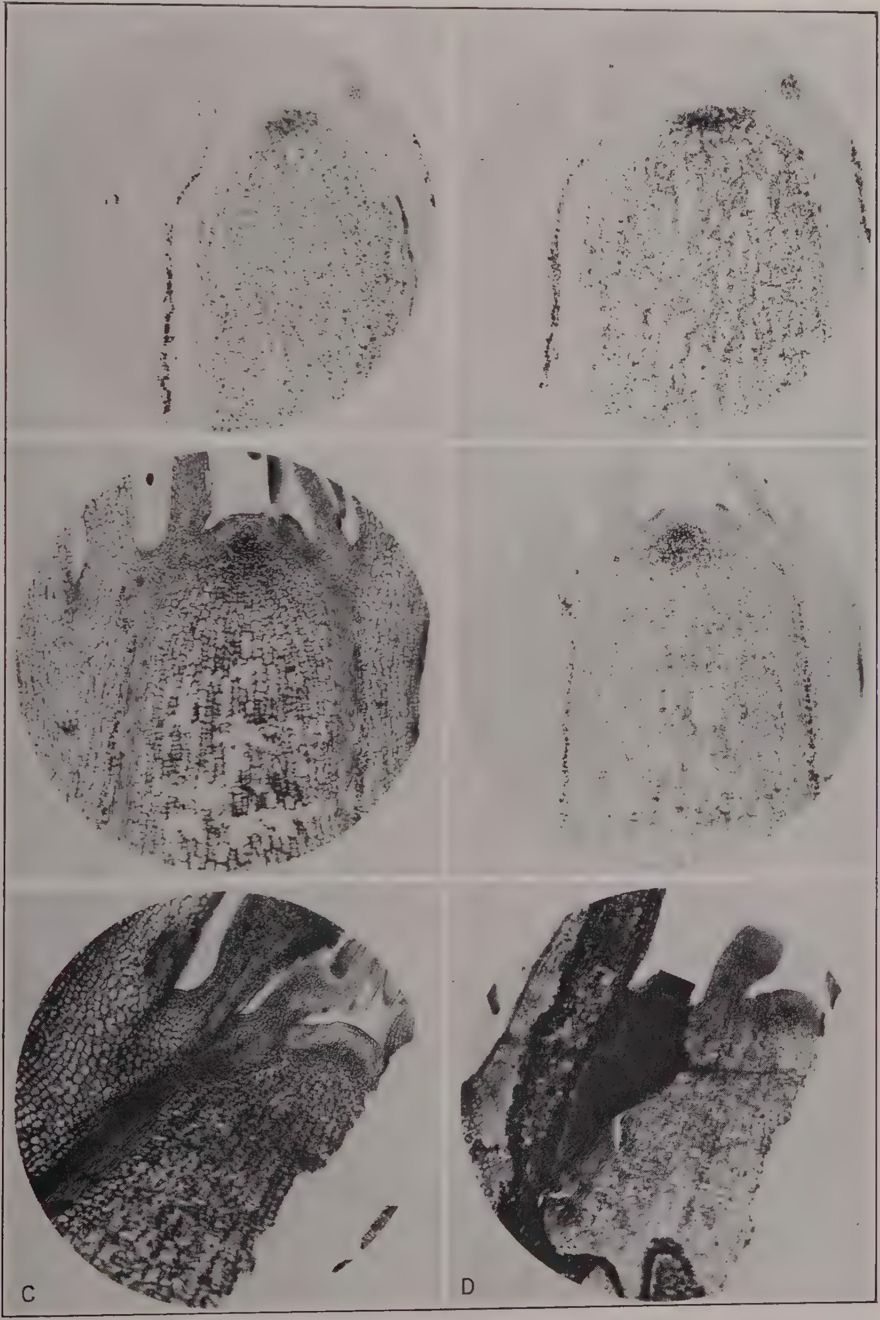


FIGURE 3 (*Cont'd.*). See legend on facing page.

(second from left), while the pectic substances were relatively low (extreme left). Apices of older normal seedlings with considerable thickening of the cell walls are also shown in the center row in Figure 1. The apex is from a 10-week-old seedling, 300 mm. in height, whose stem had ceased elongating while the tip was still green and succulent. Since hardening of the apical bud occurred shortly after this previous stage of development, the apex of the 20-week-old seedling shown in the bottom row had been dormant for a considerable period. A large amount of cellulose was present in the cell walls of both the semi-dormant and dormant shoots (extreme right, center, and bottom rows respectively), while the other wall components were also quite prominent.

The median longitudinal sections illustrated in Figure 1 extend approximately 720 μ down from the tip, while the cross sections of the stem of the normal seedlings shown in top and center rows of Figure 2 are from regions farther down the stem, that is, approximately 6,500 and 1,000 μ respectively from the tip. At this distance the walls of both the extremely succulent, rapidly-growing, three-week-old (top row) and the 20-week-old dormant (center row) seedlings contained a considerable amount of noncellulosic polysaccharides (second from right), and, in addition, the pectic materials were quite abundant in the dormant tissues (center row, extreme left).

Photomicrographs of the apical shoots of physiologically dwarfed seedlings appear in Figure 3. When germinated unchilled seeds were planted in the greenhouse, the seedlings were actively growing and approximately 10 mm. tall within a week. In the apical cell walls of these plants, as in the actively-growing normal shoots, hemicellulose was relatively abundant while the pectic substances, noncellulosic polysaccharides and cellulose, were low (top row). When the seedlings were five weeks old they were approximately 50 mm. tall, had ceased elongating, and had acquired the characteristics typical of physiological dwarfing; that is, the internodes were telescoped and many of the leaves, some of which were abnormal, were clustered in a whorl at the apex. The stems, however, were still succulent, thin, and pale green. The cell walls of such a seedling (center row), when compared with the one-week-old seedling (top row), contained slightly more cellulose (extreme right) and relatively more noncellulosic polysaccharides (second from right). The apex of a typical 10-week-old dwarf is shown in the bottom row of Figure 3. Many of the very dark green leaves of this seedling had greatly enlarged while the telescoped stem (approximately 30 mm. tall) had increased considerably in girth and appeared hardened. The apical tissues of this dormant dwarf (bottom row) resembled those of the dormant normal seedling in having thickened walls which were relatively high in cellulose content (extreme right). The pectic substances (extreme left) and the noncellulosic polysaccharides (second from right) had also increased considerably. In Figure 2, bottom row, a cross section is shown of the stem of a one-week-old actively-growing dwarf taken from a region approximately 3,350 μ from the tip. More cellulose and noncellulosic polysaccharides was present in this more mature tissue (extreme right and second from right) than farther up the stem (Fig. 3, top row).

All of the various cell wall components in the germinating embryos were present and there were no apparent differences between those which would subsequently produce normal seedlings or dwarfs. The apex shown in Figure 4 is from an unchilled embryo held five days under germination conditions, during which some expansion of the leaf primordia, slight epicotyl

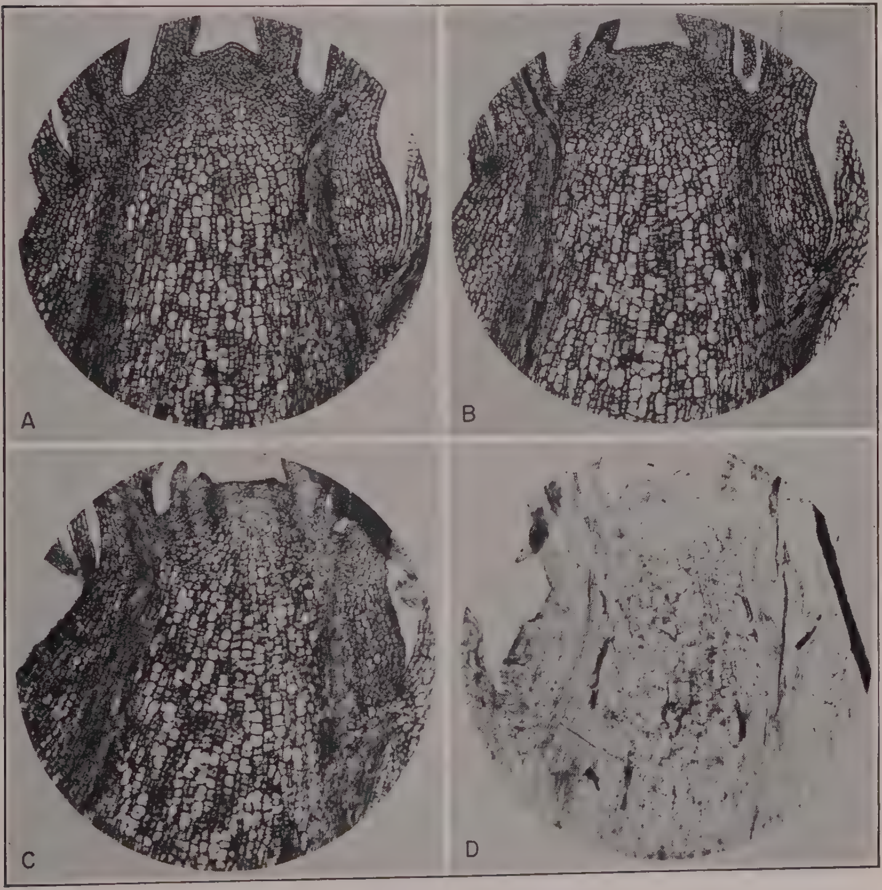


FIGURE 4. Apex of germinating unchilled seed with most of starch hydrolyzed prior to extraction. Treatment—A, B, C, D are as described for Figure 1. (×62.5)

elongation, and considerable root development had taken place. The amount of noncellulosic polysaccharides was considerably greater than either the hemicellulose or pectic substances, while the cellulose in the thin apical initials was much lower than in the other cell walls of the embryo. The apical initials in other rapidly-growing tissues as well as the provascular and cambial region in both the developing and dormant shoots, were also very low in cellulose.

Many small starch particles which stained brilliantly with Schiff's reagent (6) were sometimes either scattered throughout the pith and cortical cells, concentrated in the subapical region, or in the starch sheath and during the early stages of germination such large quantities were present that it was preferable to hydrolyze the starch prior to staining (full details will be published later).

A general change in the morphology of the cell occurred during the extraction of the noncellulosic polysaccharides from the walls of the various apices described above. The cell diameter in actively-growing material was reduced approximately 15 per cent, while the reduction was not quite as drastic in the semi-dormant and dormant apices where there was considerable thickening of the walls.

DISCUSSION

In grafting experiments with physiologically dwarfed peach seedlings it has been shown (4) that the metabolic disturbance which produces the telescoped internodes and abnormal leaves is apparently located in the shoot apex. In the present study, the various components of the cell wall of the apical shoots of both the normal and dwarfed seedlings were determined cytochemically (7), and after each of three sequential extractions the polysaccharides remaining in the walls were oxidized with Schiff's reagent (leuko-basic fuchsin) which then yielded colored compounds.

Due to the development of this technique, Jensen (7) revealed the importance of both hemicellulose and noncellulosic polysaccharides in the development of the wall in the onion root tip. In the present study with the shoot apex of peach, as in the onion root tip, reduction of cell diameter occurred when the noncellulosic polysaccharides were removed. Jensen suggested that these substances may hold cellulose under tension, while Setterfield and Bayley (10) presented the viewpoint that shrinkage in size during extraction may be due to mercerization which occurs when cellulose is subjected to a strong alkali. The extraction of the various wall components with enzymes would probably clarify this point.

In any event, it is important to interpret accurately the various intensities of color observed through the microscope, and since the range is from brilliant purplish red to pale pink this must be carefully checked with the photomicrographs. Quantitative differences were readily observed in the growing and dormant shoot apices which are described above, and the various wall components were present in all cells irrespective of the stage of cell development.

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RICHARD J. BLOCK
1906–1962

RICHARD J. BLOCK—THE MAN AND THE SCIENTIST
(May 4, 1906–February 4, 1962)

George L. McNew and Hartley W. Howard[1]

Richard J. Block was born in Macon, Georgia, on May 4, 1906, and died in an airplane crash near Tingo Maria, Peru, on February 4, 1962, while on a mission for the National Institutes of Health. In between these dates he established an enduring reputation as a biochemist and nutritional expert in the field of amino acids and proteins, contributed immeasurably to scientific progress by his discoveries and publications, and created a multitude of warm friendships among his immediate associates and world-wide acquaintances.

His career was exceptional in that he developed, as a graduate student, an area of major interest that was to be followed throughout his life. With very few exceptions, every excursion into a new area of investigation was used to gain a broader understanding of amino acid composition of proteins in relation to their biological properties and nutritive value, and the interconversion and metabolism of amino acids in healthy and abnormal individuals. Although he was a flexible individual with a far-ranging curiosity that interested him in many fundamental and applied aspects of nutrition, he persistently came back to those problems revolving around the amino acids.

After completing his undergraduate training in organic chemistry at Yale University in 1928, he enrolled in the graduate school to continue his training in the Department of Physiological Chemistry. Here he fell under the guidance of Professor Lafayette B. Mendel who exerted a life-long influence on his career. It was also his good fortune to be associated during this formative period with Dr. Herbert B. Vickery of the Biochemical Laboratory of the Connecticut Agricultural Experiment Station in several research programs. From the very inception of his career, he demonstrated decided ability to prosecute several lines of investigation simultaneously and to associate himself with others in productive collaborative arrangements so his own skills would be magnified and extended into allied fields.

After receiving his Ph.D. degree from Yale University in 1931, he spent a year in the laboratory of Professor F. von Muller of Munich, Germany, as a consulting chemist. In June 1934 he accepted a position as Research Assistant in Biochemistry at the New York Psychiatric Institute where he was promoted in 1938 to Research Associate in Biochemistry.

During the next five years his extensive experience on amino acid metabolism, methodology in analyses of proteins and amino acids, and his creative imagination prompted many individuals and industrial organizations to seek his advice. Among these was the Borden Company with which he was to maintain a constructive and rewarding association in varying capacities as consultant, employee, and informal adviser until his death. In 1943 he resigned from the Psychiatric Institute, expanded the laboratory in his home, and began a career as private consultant and free lance scientist. During this period, he made many inventions of new processes for isolating and using essential nutritional components of plants, milk, and other products.

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[1] The Borden Co., New York, N. Y.

During this period (1943–1960) he held an appointment as Professorial Lecturer at the New York Medical College, and in 1961 was designated as Visiting Professor of Experimental Pathology. During this time he actively lectured students and collaborated with the staff on medical research. In 1960 he was appointed Visiting Professor to the Department of Physiology and Biochemistry, and Associate Member of the Bureau of Biological Research at Rutgers University.

He came to the Boyce Thompson Institute in October 1951 as Nutritional Biochemist to organize a research program under the sponsorship of the Borden Company and continued on after the project was concluded in December 1955. During the next six years he organized fundamental research programs on sulfur metabolism of insects and lower plants, basic composition of serum proteins, iodine compounds of normal and goiterous individuals, and amino acid composition of wheat products. Over the same interval he served as Consultant to various individuals, UNICEF, and governmental agencies, such as the U. S. Department of Health, Education, and Welfare. It was in connection with these duties that he made the ill-fated trip to South America to advise the Nutrition Study Section of the National Institutes of Health on inter-American nutrition and to collect plants reputedly useful in suppressing cancerous growths.

His contributions from research were remarkable. As a graduate student, he soon detected a striking uniformity of amino acid composition in the keratins from human skin, sheep wool, goose feathers, snake epidermis, and silk fibroin in his research with Dr. Vickery. Histidine, lysine, and arginine were present in all of them in a ratio of 1:4:12. This led to a proposed redefinition of the keratins and was to influence his thinking in later years on the genesis of proteins from amino acids. He extended these observations to serum proteins of human, dog, and cattle where he found a constant ratio of arginine to lysine of 10:18, regardless of sex. In hemoglobin of horse, sheep, and dog he was to find a consistent ratio of iron, arginine, histidine, and lysine of 1:3:8:9.

As early as 1934 he had begun to formulate an idea that the basic amino acids formed a basic matrix or structure for proteins, for which he borrowed the term "anlage" from the German language. It is interesting to note that he had formulated a clear concept long before sequential analyses of amino acids had laid a foundation for understanding it and before the role of nucleic acids was understood at all. He believed that the other amino acids varied widely in number and amount around the three basic amino acids to give individual attributes of the members that deviate from the basic structural relationship common for a given tissue or class of a related group of organisms.

In cooperation with G. R. Cowgill and others, he took a side excursion into studying the antineuritic vitamins during the period 1931–1935. His attempts to purify and prepare highly concentrated B_1 (thiamine) may be marked down as his one acquiescence to the vitamin fad prevailing during his early years in science. They improved the methods of assay and soon showed that vitamin B was a complex mixture when efforts were made to purify it by solvent extraction, precipitation, and oxidation of impurities. The studies were extended to B_1, B_2, and G vitamins.

In 1930 he began the investigation of sulfur amino acid metabolism which was to occupy his attention for the remainder of his life. In collaboration with R. W. Jackson it was demonstrated that methionine added to a cystine-low diet improved the growth of animals. It was subsequently shown that both *l* and *d* forms of methionine, as well as the analogous alpha-hydroxy compound, could be utilized for growth. Studies with E. Brand, and others, of cystinurics shed further light on the metabolic relationships between cystine and methionine and led to the postulated intermediate formation of S-(alpha-carboxy-aminoethyl)homocysteine by a scheme which was subsequently demonstrated to be essentially correct.

It was only one step from these studies on the relation of amino acid metabolism to disease formation in cystinurics to a consideration of phenylketonuria. Dr. Block was concerned with the failure of hundreds of children born in the United States each year to metabolize phenylalanine to tyrosine. Abnormal quantities of phenylpyruvic acid accumulate in the urine and the children fail to develop normal mentality. As early as 1935 he suggested that these children could be saved if most of the phenylalanine was removed from babyfood by hydrolysis of protein followed by selective absorption of phenylalanine on activated charcoal. Some 20 years later this sensible suggestion was put into operation in an English hospital and within the past few years special diets have been made available in the United States.

His early studies on serum protein convinced him that many fallacious ideas were being generated as to the complexity of proteins because of changes induced by the drastic treatments used in salting out mixtures of materials. As early as 1932, Dr. Block became interested in physical methods of separating and purifying cell constituents. He developed a continuous extractor with an immiscible layer of solvent between two aqueous phases. This interest in methodology made him very receptive to use of paper chromatography and paper ionophoresis in protein and amino acid research when these methods were first described. As soon as the methods were reduced to routine use in his laboratory, he proceeded to prepare manuals to help his colleagues use them.

In the last decade he compiled and published, in collaboration with his associates, four reference books and laboratory manuals that came to be looked upon as standard reference sources around the world. These were "The Amino Acid Composition of Proteins and Foods" (with Diana Bolling); "Amino Acid Handbook. Methods and Results of Protein Analysis"; "A Manual of Paper Chromatography and Paper Electrophoresis" (with Emmett L. Durum and Gunter Zweig); and "A Laboratory Manual of Analytical Methods of Protein Chemistry. Vol. 3" (with P. Alexander). During this period he also wrote no less than eight chapters in as many books by other authors. At the time of his untimely death he was in the final stages of editing another book and had plans well advanced for compiling a second one by various authors. These books, 128 original and review publications in the scientific literature, and more than a score of patents remain as his legacy to biochemistry.

Although Dr. Block left a record of unusual scientific achievement in his career, he will be best remembered by his associates for his stability of character, steadfastness, and purposefulness. He was a kind, considerate, and tolerant individual with great capacity, and desire, to work with others. He dealt most considerately with his assistants, constructively and generously with his contemporary colleagues, and even with tolerance for the administrators who simply could not ensnare him in their red tape. He had great loyalty for his associates, his sponsoring institutions, and his assistants. Needless to say, he left many to mourn his passing.

The year before he competed his graduate training he married Margaret Strasser, a young educational psychologist. She ran an efficient household where warmth of love and strength of personality could flourish. Eventually they were to die together after almost 32 years of marriage. Two daughters, Mrs. Werner (Marion) Krebser and Mrs. Thomas C. (Diane) Montie, and four grandchildren survive them.

Among other organizations, Dr. Block was a member of the American Society of Biological Chemistry, the American Chemical Society, the American Institute of Nutrition, the Institute of Chemists, the Biological Society (Great Britain), the Society for Experimental Biology and Medicine, the Harvey Society, the Chemists Club, Sigma Xi, and was a fellow of the American Association for the Advancement of Science and the New York Academy of Sciences.

VOLATILE ATTRACTANTS FROM PONDEROSA PINE ATTACKED BY BARK BEETLES (COLEOPTERA: SCOLYTIDAE)[1]

J. P. VITÉ[2] AND R. I. GARA[3]

SUMMARY

A specially designed apparatus blowing air from a box containing test material into a funnel-shaped trapping device was used in the forest to test the response of the scolytid populations to volatile materials emanating from log sections of ponderosa pines, *Pinus ponderosa* Laws., artificially infested with bark beetles of chosen sex and species. The attractive material responsible for mass attack of ponderosa pine by *Ips confusus* (Lec.), *Ips ponderosae* (Sw.), and *Dendroctonus brevicomis* Lec. was found to be species specific and to be produced in suitable breeding material by mature male beetles in both species of *Ips* but by mature female beetles in the *Dendroctonus* species. In contrast to leading theories, there was further evidence that microorganisms associated with bark beetles such as yeasts were not involved in the production of attractants, while raw oleoresin tapped from ponderosa pines proved attractive to several other coleopterous species of secondary importance only.

Distinct diurnal and local patterns in the response of the bark beetle populations to attractive materials were found to be temperature dependent. The attracted populations concentrated closely towards the sources of attractants without noticeable increase of the population level in the general area. In these experiments, the random sampling of the aerial populations was accomplished by rotary net traps.

The attractiveness of the test material was found to depend on the number of beetles used in the infestation and on the age of infestation. It was possible to intensify the attraction by treatment of the log sections with volatile solvents such as gasoline or kerosene prior to infestation. Experimental release of *Ips confusus* marked with a fluorescent dye and their subsequent recovery within 2 to 50 minutes over distances between 10 to 600 meters suggested the efficiency of the attractants over long distances.

It is concluded that the materials responsible for mass attack of ponderosa pine by *Dendroctonus brevicomis* as well as *Ips confusus* and *Ips ponderosae* are species specific, sex depending attractants produced by mature female and male beetles, respectively, in contact with suitable host material. The fact that these attractants are efficient over long distances when intensified mechanically or chemically suggests their possible value in bark beetle control and population studies.

INTRODUCTION

Although the use of trap trees has been a common and efficient practice in bark beetle control for more than a century, little is known about the

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origin and nature of the attractive forces they so obviously possess (8, 14, 19, 20, 21, 26). It has been fairly well established, however, that bark beetles may find suitable host material primarily in response to chemotactic stimuli (10, 18, 23). There exist four general explanations as to the origin of the attractant: either trees in subnormal physiological condition attract bark beetles *per se* as has been widely assumed by earlier workers but never demonstrated (18, p. 91), or the attractiveness is a result of the successful attack of single bark beetles in a susceptible tree, as convincingly shown for the first time by Anderson (3). A combination of both, the third explanation suggests an initial attraction of trees in subnormal health followed by a stronger, secondary attraction as a result of the invasion by a few pioneer beetles (16). A fourth explanation assumes that host trees are simply recognized by specific odors such as resinous vapors and attacked randomly (7). Most explanations agree that the attractants are probably volatile substances which, as a rule, originate in susceptible trees (1, 2, 4, 13, 15, 25).

In fact, a review of literature suggests that in spite of similar life habits all of these explanations may apply to the relationship between certain bark beetles and certain host trees. The explanation that weakened trees are more attractive to bark beetles *per se* than trees of normal physiological condition may prove true for Norway spruce (*Picea excelsa* L.) (14, 17), under attack by *Ips typographus* L. It certainly does not apply to *Pinus banksiana* Lamb. under attack by *Ips pini* (Say.) and *Pinus ponderosa* under attack by *Ips confusus* where random attack by single beetles was found to be the only source of attraction (3, 27).

It has been shown experimentally that *Ips confusus* comes in large numbers to logs and trees after a few male beetles were successfully established. The question remained where the attraction originated and whether the beetles responded actually to volatile materials *per se* or together with some stimulation other than olfactory. Therefore, a study of the chemotropic responses of the bark beetle population under field condition was undertaken. To avoid visual, thigmotactic and other reactions of the beetles which might interfere with olfactory responses, air was drawn from the assumed sources of attractants and tested in devices that would permit trapping insects as they respond to the air. Thus, by separating the source of attractants and attractive substance under continuous bioassay, it was hoped to separate the material actually responsible for the mass attraction of ponderosa pine bark beetles.

MATERIALS AND METHODS

Prerequisites for the continuous bioassay of attractants under field condition are efficient trapping devices and a simple method of sampling the bark beetle populations in flight. Satisfactory results were obtained by using two devices and a combination of the two: aerial net traps for the random collection of insects in flight and funnel-shaped field olfactometers for selective trapping. Each apparatus was standardized for optimal efficiency.

Aerial net trap. Aerial nets 38 centimeters in diameter and 70 centimeters in depth were suspended on pipes 234 centimeters long and driven by a ¼ horse power electric motor of 60 r.p.m. (Fig. 1). A conventional shaft coupler, which utilized a rubber plate, connected the net arms with the motor shaft for instant assembly and removal of the net. The motor was hung by two L-shaped supports from the top of an aluminum ladder of 2 or 3 meters length. The whole device could be easily handled and transported by one person. Each net traveled approximately 53 kilometers per hour in a radius of 253 centimeters, sampling approximately 6,000 cubic meters of air.

The shape, radius, and speed mentioned above proved to be the most efficient in trapping bark beetles in flight (23), giving a true figure of their aerial population density (9). Nets with elongated bags of medium mesh screen represented less air resistance as well as less damage to or loss of small insects than shorter bags with finer screens (5). Speeds below 53 kilometers per hour and radii of less than 200 centimeters caught fewer insects.

Field olfactometer. The olfactometer developed for field observations consisted of three parts: a box 60 centimeters in diameter containing the test material or chemical, a funnel 100 centimeters deep and 60 centimeters in diameter, and a pipe section with a fan 30 centimeters in diameter which connected the box with the funnel. Air was drawn from the box and blown through the lower third of the funnel which was made of metal screening. Glass or plastic windows set across the top of the funnel served for better distribution of the air saturated with attractant as well as a barrier to flying insects.

A photograph of this apparatus is presented in Figure 2. The base, pipe section, and outer funnel of the olfactometer were made of 22 gauge sheet metal sections that fitted easily into one another, thus allowing for quick assembly or dismantling.

The device was designed to facilitate transportation as well as adaptation to different uses. In addition, a removable ring containing 8 holes of 10 centimeter width covered by screens served for ventilation either as the bottom unit of the olfactometer or as the top unit of a supplementary tank. This tank was connected by a 15 centimeter pipe to the bottom unit of the olfactometer when larger log sections were used in the experiment.

The pipe section connecting box and funnel contained a ¼ horse power electric motor rated at 1,500 revolutions per minute producing a maximal air volume of approximately 25 cubic meters per minute with an air speed

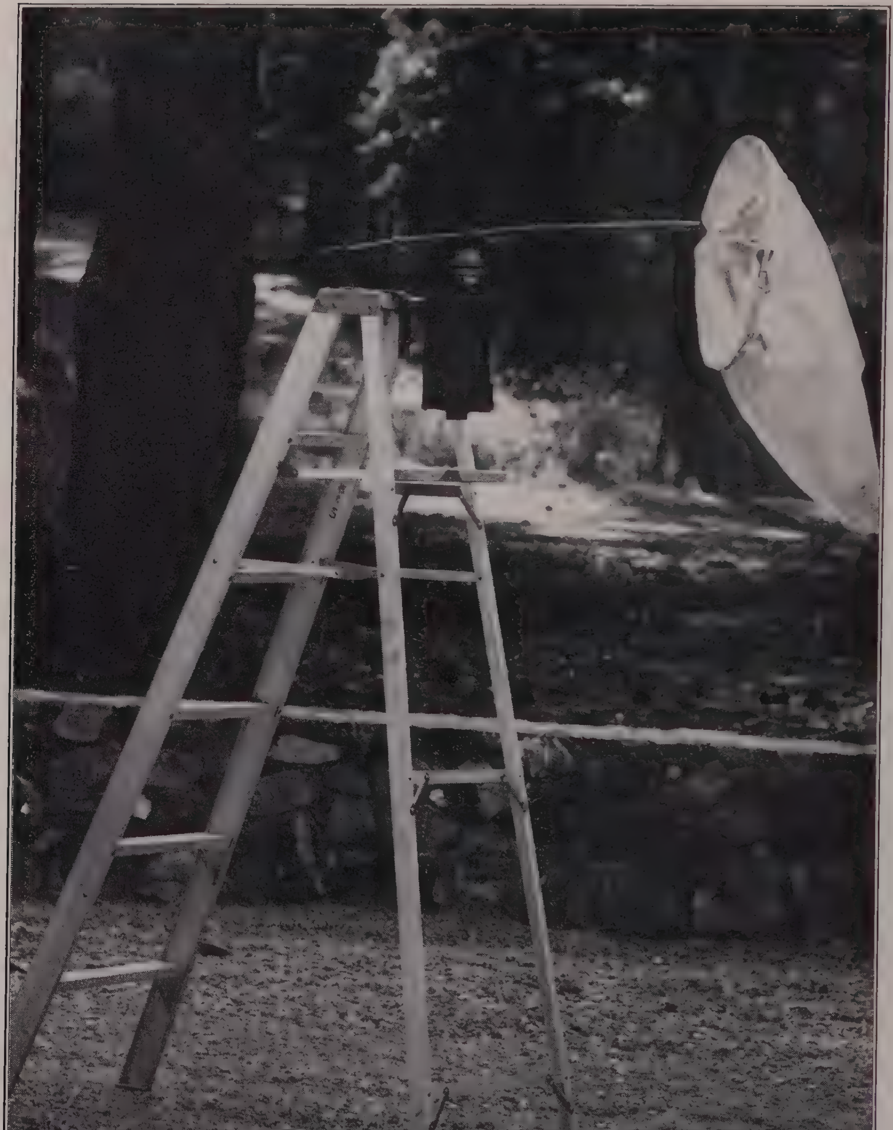

FIGURE 1. Rotary net trap attached to an aluminum ladder.

of 120 meters per minute. Air volume and speed were regulated either by a butterfly valve installed below the fan, reducing the air speed to 30 meters per minute, or by a ring closing the ventilation holes at the base of the box (Fig. 2). Immediately below the fan, a short nipple 0.6 centimeter in diameter provided an inlet for air saturated with test material when attractants were blown from distant sources through plastic pipes. The nipples were also used for the attachment of flow gauges.

The upper two-thirds of the funnel was made from sheet metal, while the lower third (covered by the pipe section) was constructed of metal screening that pointed, with a 4 centimeter wide opening, into a collecting jar. A sliding door in the pipe section facilitated the removal of the mason jar affixed to the screen portion of the funnel by means of a screw ring.

In operation, the attracted insects flew against the glass barrier, or entered the funnel by falling or crawling into the glass jar after resting a shorter or longer period on the inside of the funnel. Since predators were as much attracted as bark beetles, it became necessary to add some water into the glass jar to avoid losses from predation or escape when checks were made less frequently than each hour.

The described apparatus resulted from experimentations with similar

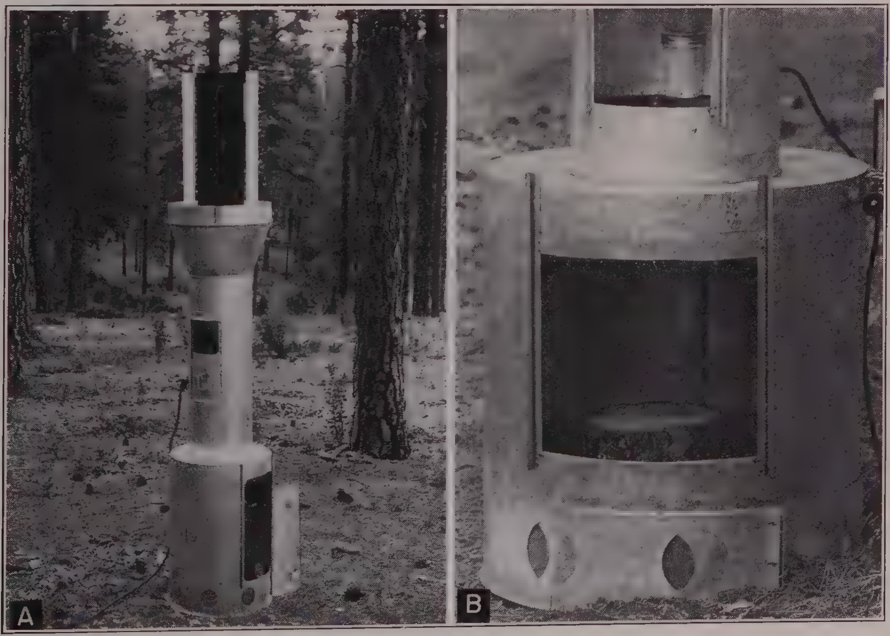

Figure 2. Field olfactometer. (A) Black and white painted glasses were used in experimentation with *Dendroctonus valens*. (B) Lower portion of the olfactometer showing the box containing the test log and the mason jar affixed to the screen funnel.

designs (23) in which the primary limiting factor in attracting or trapping insects proved to be the air stream produced by the fan. The most efficient air speed varied with body weight and flight capacity of the species tested. However, with an increasing air velocity more insects, especially small ones, were prevented from entering the funnel. A comparative study in attracting three coleopterous species (*Dendroctonus brevicomis*, *Hylastes macer* Lec., and an undetermined bostrychid) to four olfactometers of various air stream speeds revealed the relationship presented in Figure 3. Best results for these three species of similar size were obtained with air blowing at a rate between 60 and 90 meters per minute. In similar tests with much larger beetles such as *Dendroctonus valens* Lec., however, best results were obtained with air currents between 90 and 120 meters per minute.

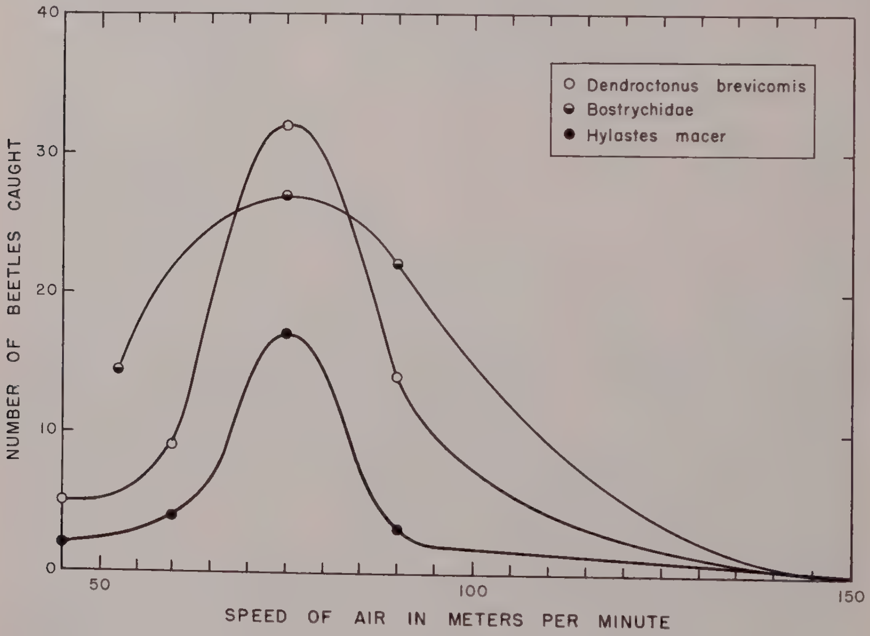

FIGURE 3. Influence of air movement produced by the fan on the number of insects caught in a field olfactometer during a 10-hour test.

As a secondary factor, size and color of the glass barrier on top of the funnel influenced the efficiency of the field olfactometer. Olfactometers with no barrier at all were less effective than those with one glass but olfactometers with one glass of full size together with two half-sized glasses set in a cross on top of the funnel proved superior to either. Besides doubling the barrier area, the cross arrangement provided better air distribution than either the funnel lacking any glass or with merely one.

While *Dendroctonus brevicomis* and the *Ips* species were caught without significant differences in olfactometers equipped with painted and unpainted glass, *Dendroctonus valens* responded better to a glass barrier which was painted with black and white stripes. This observation is in accordance with similar findings made by Chapman and Kinghorn (6) where dark surfaces were preferred by *Dendroctonus valens* but not by any of the other scolytids caught with the barrier traps. Since the visual sense of scolytid beetles is known to be poorly developed, they likely recognize light reflection intensities rather than colors. Therefore, dark surfaces are recognized better when placed in contrast with light colors (10).

Location of experiments. The experiments were carried out during the season of 1961 in the Boyce Thompson Institute's experimental forest at Grass Valley, California, where a special system of water pipes and power lines provides outside-laboratory facilities throughout an 800 acre stand of second growth ponderosa pine. In this forest, *Dendroctonus brevicomis*, *Dendroctonus valens*, *Ips confusus*, *Ips ponderosae*, and *Ips latidens* (Lec.) occur on an endemic population level but yet at appreciable numbers. Comparative observations were made in the Crystal Bay area at Lake Tahoe, Nevada, where *Dendroctonus brevicomis* and *Dendroctonus monticolae* are present at epidemic levels (24).

Preparation of attractive material. Throughout this study, small log sections of freshly cut ponderosa pines infested with the test insect served as bait. In general, 10-inch log sections from trees of 10- to 12-inch diameter at breast height were infested with 25 insects by placing each beetle in a hole made in the outer bark layer with a nail of adequate size. A small piece of metal screen, affixed to the bark surface with two staples, enclosed the beetle but did not hamper the discharge of boring dust and volatile material. Usually one, occasionally up to four, such log sections were placed as bait in the box of the field olfactometer.

In tests where more than one olfactometer was operated for comparative purposes it became necessary to use comparable amounts of attractive material. This was accomplished by using logs of similar size infested at the same date with the identical number of beetles. Where possible, however, all of the attractive materials were placed preferably in a central box equipped with an air compressor. From here, the air stream carrying the attractant was distributed through plastic tubing to each olfactometer and, if necessary, the distribution was regulated by flow gauges. An electrical air compressor (integral oil-less diaphragm type) of $\frac{1}{4}$ horse power producing 30 pounds pressure per square inch proved satisfactory to supply 6 olfactometers.

Marking of beetles. The use of fluorescent dyes to mark individual beetles was preferred to the conventionally used amputation of tarsi, other marking dyes, or radioactive tracers. This allowed the instant recovery of the marked beetles caught in the glass jar of the field olfactometer, regardless of how many other beetles were caught at the same time. It also had the advantage that beetles marked with different colors could be used at the same time. A slight disadvantage was the fact that each beetle had to be treated individually and that handling and marking seemed to affect some of the beetles. The marking was done best on the pronotum with a very small amount of dye applied by a pin-pointed brush. Marking the elytrae seemed to influence the beetle's capacity to fly. It was advisable to mark a larger number of beetles than needed in an experiment. As a rule only one-half to two-thirds of the treated beetles took off readily when stimulated to fly.

PATTERN OF RESPONSE TO ATTRACTANTS

All of the beetle species investigated responded to olfactometers baited with attractants. However, it became quite evident that several factors influenced the pattern in which these bark beetles responded to sources of attractants. There were distinct differences in response to the diurnal and seasonal periods as well as in various localities and stands with high or low population levels. Under epidemic conditions, usually a relatively small portion of the population in flight was attracted presumably because of the strong competition provided by extensive sources of naturally occurring attraction. For the same reason, under endemic conditions, fewer beetles seemed to be attracted toward the end of the flight period and with the progressing season. Diurnal and local response patterns, however, seemed to be primarily dependent on temperature.

Diurnal pattern. During flight periods the response of the bark beetle populations to field olfactometers showed a definite diurnal pattern (9). Under the climatic conditions of the experiments, flight began in the early morning as soon as the temperature reached 18° to 20° C. At temperatures above 28° to 30° C., the flight activity of most species decreased rapidly. The beetles regained a flight maximum during the late afternoon and towards the evening. However, there were distinct differences as to the time of maximum response among the various species (Fig. 4). Therefore, comparable data were obtained only during two 4-hour periods of the morning (7:00 to 11:00 A.M.) and the evening flight (3:00 to 7:00 P.M.). Usually, observations were made by expanding each experiment over a period of three days, interchanging the test material between olfactometers in various positions which is a standard procedure in testing attractants to avoid side effects from various localities (12).

Local pattern. The olfactometers were always set up in the shade of trees, because high temperature, especially direct exposure to sunlight, seemed to influence the flight behavior and flight pattern. The fact that olfactometers within a pine stand consistently caught larger numbers of beetles may be attributed to the avoidance of direct sunlight (9). Correspondingly, olfactometers caught the least number of insects in openings and, furthermore, little flight activity occurred in the sun-exposed upper crown region. Traps lifted by a balloon into the upper crown region, and supplied with an attractant-saturated air stream through plastic tubing, failed to attract as many beetles as a similar olfactometer near the ground.

Pattern of concentration due to attraction. From field observations it is well known that recently infested trees, whether they are cut or standing, are frequently responsible for the subsequent attack of neighboring trees either by the same or the following bark beetle generation as a result of an increased population level due to mass attraction. It is not entirely understood, however, whether this phenomenon is a result of the limited brood material represented by the originally infested tree, or of a host selection habit of bark beetles. In view of a possible application in bark beetle control, it was determined whether the bark beetle population attracted to olfactometers led to an increase of the population level throughout the general area in which the testing took place.

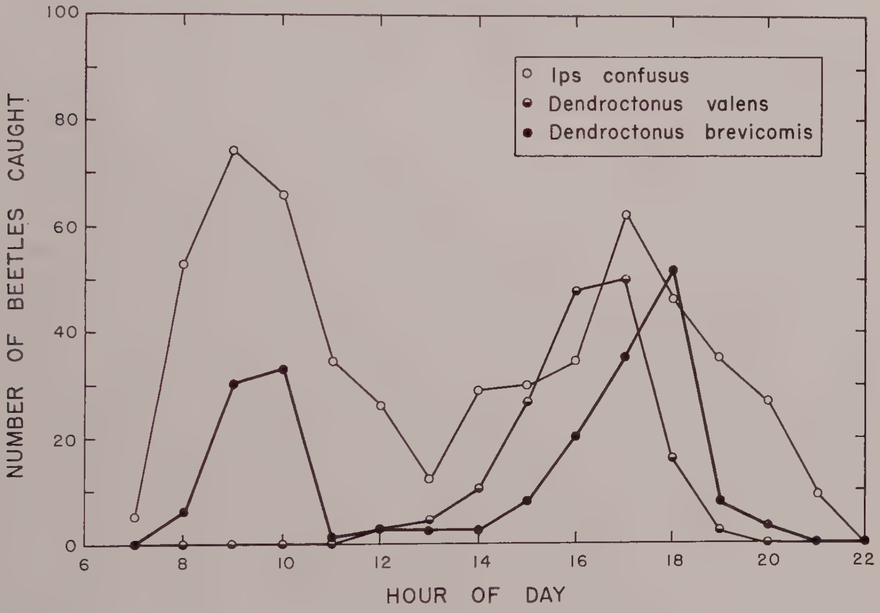

Figure 4. Diurnal flight pattern of *Dendroctonus brevicomis*, *D. valens*, and *Ips confusus.*

For this purpose, air was blown from three boxes containing log sections heavily infested with *Ips confusus* towards an aerial net rotating directly above the outlet of these boxes. Four additional rotary nets were set in an inner circle with a radius of 15 meters and another four rotary nets in an outer circle with a radius of 30 meters around this center of attraction. The result of this test is shown in Table I. Eighty-five per cent of all beetles were caught at the center, 10 per cent in the inner circle and only 4 per cent were caught in the outer circle 30 meters away from the center. These values confirmed observations made in the field. The attacking bark beetle population concentrated its flight directly toward the three boxes in the center, approaching them preferably against the prevailing wind direction. In other words, the flight focused directly on the source of attraction and an increased flight activity could not be noticed outside the 30 meter circle.

TABLE I

NUMBER OF IPS CONFUSUS CAUGHT BY ROTARY NETS AT THE CENTER AND PERIPHERY OF A SOURCE OF ATTRACTION (JUNE 29, 1961)

Time of day	Wind direction and speed, m.p.h.	Center	Number of insects caught at							
			Inner circle 15 meters from center				Outer circle 30 meters from center			
			NW	NE	SE	SW	NW	NE	SE	SW
2–4 P.M.	S 4	45	3	1	0	4	1	2	1	2
4–6 P.M.	S 4	320	22	11	7	10	2	5	4	0
6–8 P.M.	NE 3	700	11	22	26	17	2	10	12	4
Subtotal		1,065	36	34	33	31	5	17	17	6
Total		1,065 (85%)	134 (11%)				45 (4%)			

SOURCE OF ATTRACTANTS

In previous studies it has been shown that volatile materials emanating from infested logs were responsible for the attraction of *Ips confusus* and *Dendroctonus brevicomis* and that these volatile materials can be drawn with an air stream from such logs and translocated to trapping devices (23). In addition, the source of attraction was localized for *Ips confusus* (27). In this species, only the mature male beetle seemed to be responsible for attraction of both sexes while boring in the phloem tissue of suitable breeding material. Neither female beetles, teneral adult males, nor infested logs from which the males were removed proved to be attractive in the field.

These findings were repeated and broadened through application of field olfactometers (Table II). In these experiments, 6 field olfactometers were set up in a line 15 meters apart in the shade of a 90-year-old ponderosa pine stand. The possible influence of the position of each individual olfactometer was eliminated by interchanging the test material in 4-hour periods. Generally, each test was completed during three consecutive days.

The results confirmed that only logs infested with mature males of *Ips confusus* became attractive while untreated logs or logs infested with only female beetles were not attractive. Mature male beetles alone or homogenized with fresh phloem tissue were not attractive. Similarly, logs previously infested with mature males lost their attractiveness upon removal of the male beetles. In contrast to field observations on trap trees (27), however, there was still a certain residue of attractants within the first 6-hour period following the removal. To find whether this residue was located in the phloem portion or xylem portion of the nuptial chamber, both portions were separated in another experiment. The tests showed that more volatile material emanated from the xylem portion than from the phloem portion when the nuptial chamber was separated.

TABLE II

Number of Ips confusus and Ips ponderosae, Respectively, Caught in Field Olfactometers during 24 Hours on Three Subsequent Days Using a Variety of Attractant Materials

Bait (*Ips confusus* and *Ips ponderosae*, respectively)	Number of beetles caught in 24 hours	
	Ips confusus	*Ips ponderosae*
Fresh log infested with 25♂♂ (=control)	57	23
Fresh log infested with 25♀♀	0	0
Fresh log treated with cycloheximide, 150 p.p.m., and stove oil and infested with 25♂♂	86	—
Fresh log infested with 25♂♂ and 2♀♀ per ♂	56	—
25♂♂ smashed on blotting paper soaked with alcohol	0	0
250♂♂ homogenized with fresh phloem	0	0
Fresh log infested with 25♂♂ (=control)	108	14
Fresh log not infested	0	0
Fresh log infested with 25 teneral ♂♂	0	0
Fresh log infested with 25♂♂ excised from brood tree	12	6
25♂♂	0	0
Fresh log infested with 25♂♂ (=control)	60	29
Fresh log not infested	0	0
25♀♀	0	0
Fresh log with 25 nuptial chambers, ♂♂ removed	4	0
Xylem with nuptial chambers of 25♂♂ (beetles removed)	3	0
Phloem with nuptial chambers of 25♂♂ (beetles removed)	0	0

Teneral adults of *Ips confusus* when inoculated into a log did not create attraction nor did they excavate a nuptial chamber. Young, darkened adults, however, excised from brood galleries a few days before natural emergence did excavate a nuptial chamber immediately upon transfer to a fresh log but did not create attraction before another couple of days.

Of interest in connection with the suggestion by Person (16), that yeasts associated with bark beetles may produce attractants, is the fact that mature male beetles of *Ips confusus* and female beetles of *Dendroctonus brevicomis* were able to produce attraction in logs where the growth of

TABLE III

NUMBER OF DENDROCTONUS BREVICOMIS CAUGHT IN FIELD OLFACTOMETERS DURING 24 HOURS ON THREE SUBSEQUENT DAYS USING A VARIETY OF POSSIBLE SOURCES OF ATTRACTION

Bait	Number of beetles caught in 24 hours
Fresh log infested with 25 ♀ ♀ (=control)	37
Fresh log infested with 25 ♂ ♂	0
Fresh log not infested	0
Fresh ground phloem and 25 ♀ ♀	0
25 ♀ ♀	0
Fresh log infested with 25 ♀ ♀ and 25 ♂ ♂ separately (Crystal Bay)	6
Fresh log treated with cycloheximide 150 p.p.m. and stove oil and infested with 25 ♀ ♀ and 25 ♂ ♂ separately (Crystal Bay)	52

yeasts was inhibited by soaking the logs in cycloheximide (150 p.p.m.) and stove oil (low grade kerosene) prior to infestation.

Identical tests performed with *Ips ponderosae* did confirm that the source of attraction must be similar to that of *Ips confusus* while tests with *Dendroctonus brevicomis* clearly indicated that the opposite sex, the mature female beetles, was responsible for the attraction under otherwise similar conditions (Table III).

SPECIFICITY OF ATTRACTANTS

To study the specificity of the volatile materials emanating from infested logs, tests were conducted with controlled infestations of the various bark beetle species throughout the 1961 season. The results of these tests are presented in Tables IV and V. They indicated the existence of several

TABLE IV

NUMBER OF BARK BEETLES IN FLIGHT ATTRACTED TO OLFACTOMETERS CONTAINING LOGS INFESTED WITH DENDROCTONUS BREVICOMIS (D.B.), D. VALENS (D.V.), IPS CONFUSUS (I.C.), IPS PONDEROSAE (I.P.), IPS GUILDI (BLKM.) (I.G.)

Logs infested with	Date of test	Time of test in hours	Number of beetles caught				
			D.b.	D.v.	I.c.	I.p.	I.g.
Dendroctonus brevicomis	May 13–June 11	60	519	89	0	1	2
Dendroctonus brevicomis	July 27–31	14	38	9	0	0	0
Dendroctonus monticolae	May 23–25	10	9	3	0	0	0
Ips emarginatus (Lec.)	May 13–15	20	2	56	6	4	11
Ips confusus	May 13–15	12	0	12	9	2	8
Ips confusus	June 25–July 1	32	2	26	1,673	*	*
Ips confusus	July 23–29	32	1	8	176	1	0
Ips ponderosae	July 23–29	24	0	2	5	49	1
Uninfested logs	May 8–June 11	24	1	97	1	0	0
Oleoresin (diluted with alcohol)	May 17 and 18	4	1	138	1	0	2

* Not in flight during experimental period.

TABLE V

RESPONSE OF BARK BEETLE POPULATIONS IN FLIGHT TO VARIOUS MATERIALS

Bait material placed in olfactometer	Insect species caught in traps*												
	Ips ponderosae (Sw.)	*Ips confusus* (Lec.)	*Ips guildi* (Blkm.)	*Dendroctonus valens* Lec.	*Dendroctonus monticolae* Hopk.	*Dendroctonus brevicomis* Lec.	*Hylastes macer* Lec.	*Hylastes nigrinus* (Mann.)	*Hylastes minutus* Blkm.	*Pityophthorus* sp.	*Xyleborus scopulorum* Hopk.	*Temnochila virescens* (Mann.)	*Enoclerus lecontei* Wolc.
Sections of ponderosa pine log recently infested with:													
Ips emarginatus	±	±	±	+	—	—	o	o	o	o	o	+	+
Ips ponderosae	+	±	±	+	—	—	o	o	o	o	o	+	+
Ips confusus	±	+	±	+	—	—	o	o	o	o	o	+	+
Dendroctonus monticolae	—	—	—	+	+	±	o	o	o	o	o	+	+
Dendroctonus brevicomis	—	—	—	+	±	+	o	o	o	o	o	+	+
Sections of ponderosa log with advanced infestation of:													
Ips emarginatus	±	±	±	+	—	—	+	+	+	o	o	+	+
Ips ponderosae	+	±	±	+	—	—	+	+	+	o	o	+	+
Ips confusus	±	+	±	+	—	—	+	+	+	+	o	+	+
Dendroctonus monticolae	—	—	—	+	+	±	+	+	+	o	o	+	+
Dendroctonus brevicomis	—	—	—	+	±	+	+	+	+	—	o	+	+
Uninfested sections of ponderosa pine log	—	—	—	+	±	—	+	+	o	o	o	±	o
Frass of *Dendroctonus monticolae* and *Dendroctonus brevicomis* and alcohol	—	—	—	—	—	—	o	o	o	o	+	+	o
Acetone	—	—	—	—	—	—	o	o	o	o	o	o	o
Turpentine	—	—	—	±	—	—	o	o	o	o	o	o	o
Alcohol (ethyl-)	—	—	—	—	—	—	o	o	o	o	o	o	o
Oleoresin	—	—	—	+	—	—	o	o	o	o	o	o	o
Oleoresin + alcohol	—	—	—	+	—	—	o	o	o	o	+	±	o
Oleoresin + acetone	—	—	—	+	—	—	o	o	o	o	o	o	o
Oleoresin + turpentine	—	—	—	±	—	—	o	o	o	o	o	o	o

* Relative number of beetles in traps: + = positive response; ± = occasional response; — = no response; o = not present during test.

major groups of attractants to which bark beetles, other subcortical feeding insects, and their predators responded. Obviously, the host selection behavior varied with ecological groups. Thus, the attractive material for the group of bark beetles capable of primary attack, i.e., *Dendroctonus brevicomis*, *Ips confusus*, and *Ips ponderosae*, proved to be species specific. Material infested with *Dendroctonus* did not attract *Ips* species beyond a random chance level, nor did material infested with *Ips* attract *Dendroc-*

tonus, and only occasional responses occurred within the same genus. This may indicate the existence of closer affinity among the attractants produced by the same genus than among those produced by beetles of different genera.

Another group of insects, especially *Dendroctonus valens* and *Xyleborus scopulorum* Hopk., responded to fresh logs whether they were infested or not. Further tests, involving these species, proved that raw oleoresin alone included the source of attractants. Resin alone or a combination of resin together with fermented phloem proved to be attractive to a number of secondary ponderosa pine insects such as *Hylastes* spp., several Buprestidae, Cerambycidae, and Bostrychidae.

Also, predators, especially *Temnochila virescens* var. *chlorida* (Mann.) and *Enoclerus lecontei* Wolc. and, to a lesser degree, *Enoclerus sphegeus* (Fabr.) responded to both infested and uninfested logs. However, they responded to infested logs far more frequently than to uninfested logs. As an exception, a small bostrychid species consistently responded to olfactometers in which ethyl alcohol had been used.

INTENSITY OF ATTRACTION

Important for experimentation as well as for the possible application of attractants in bark beetle control is the determination of their attractive power. It is necessary to compare various sources of attraction influenced by the number of attacks and the age of the infestation. For field application it is necessary to know whether the attractiveness of recently attacked trees can be overcome by the attractants and over what distances such attractants prove effective.

Number of attacks. The relationship between the number of attacking beetles and their attractive power was studied for *Ips confusus* in two series of tests. Fresh log sections of 9- to 12-inch diameter and 24 inches in length cut from the same tree were infested with various numbers of mature male beetles ranging in one series from 1 to 6 and in another from 6 to 100. Then each of the logs was placed in each of six field olfactometers for a 4-hour period. The results are presented in Figure 5. They indicated a rapid increase in attractive power when the number of attacks by male beetles increased above two. This phenomenon is in keeping with field observations (3, 24) where mass attack occurred as soon as a few beetles were successfully established (27, Fig. 1).

However, both series exhibited the rapid increase on different levels. The series using only 13 beetles as bait in all tests drew a total of 62 *Ips confusus*, the series using 193 beetles as bait drew 7,948 in three 8-hour periods of three subsequent days (July 1 to 3, 1961). This test indicates that the beetles must have been attracted from a considerably larger area.

Age of infestation. With temperatures above 24° C., logs infested with

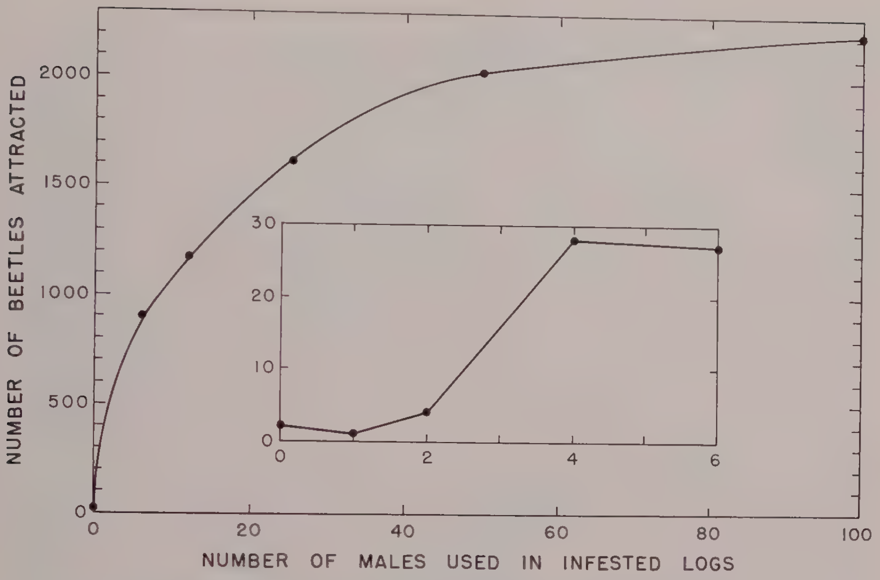

Figure 5. Influence of the number of *Ips confusus* males implanted on the attractive power of artificially infested logs.

mature males of *Ips confusus* became increasingly attractive six hours after inoculation (Table VI).

There was an obvious coincidence between the beginning of attraction and the beginning of nuptial chamber construction. In general, the adult male of *Ips confusus* completed the entrance hole and reached the cambium layer for construction of the nuptial chamber six hours after inoculation (Fig. 6). A nuptial chamber of approximately 25 square millimeters was normally completed in another four hours which reached normal shape

TABLE VI

Relationship of Time after Invasion of Ponderosa Pine Logs by Ips confusus to Attraction of Free-Flying Beetles

Time of day	No. of beetles attracted to traps with		
	Freshly infested log, 25♂♂*	Log infested with 25♂♂ beetles for 3 days	Beetle-free logs (control)
12– 2 P.M.	0	2	0
2– 4 P.M.	0	7	0
4– 6 P.M.	2	16	0
6– 8 P.M.	10	27	0
8–10 A.M.	7	18	0
10–12 A.M.	36	39	0

* Logs exposed to beetle attack at 12 noon on first day of test. Records were taken, therefore, successively at 0–2, 2–4, 4–6, 6–8, 20–22, 22–24 hours after infestation.

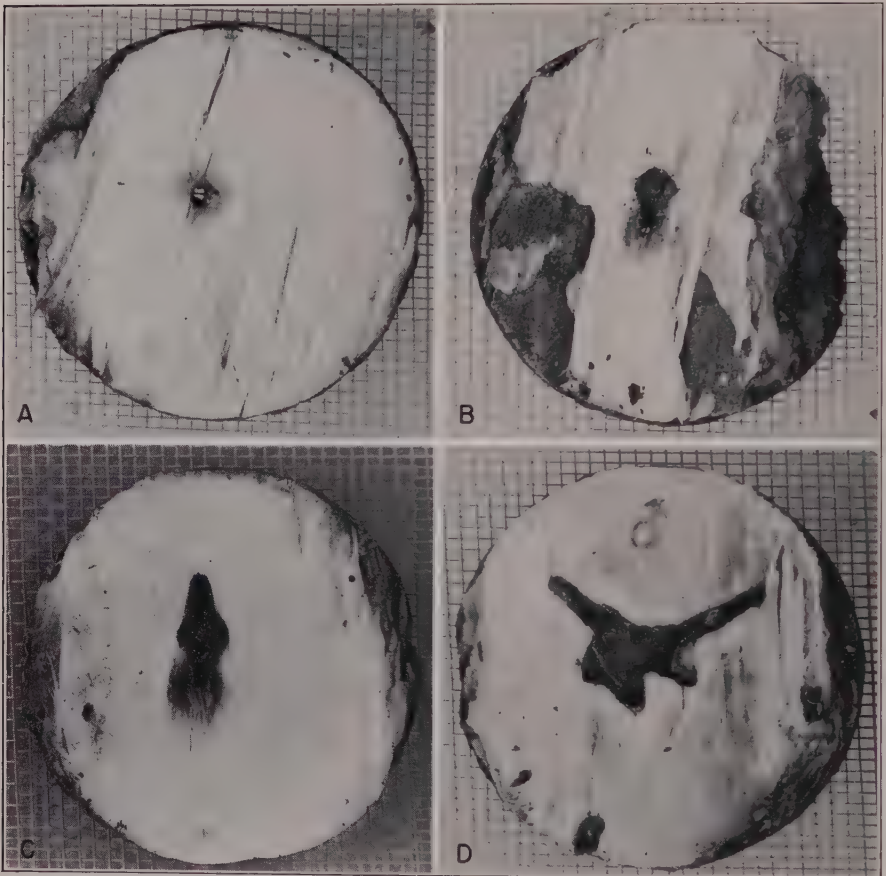

FIGURE 6. Typical size and form of nuptial chambers of *Ips confusus* 6 hours (A), 10 hours (B), and 48 hours (C) after implantation compared with a 48-hour-old nuptial chamber of *Ips ponderosae* (D).

and size of about 45 square millimeters within 24 hours after the beetle entered the log (Fig. 6). The nuptial chamber was enlarged during each following day by slightly more than one-tenth of its original size, reaching an average of 90 square millimeters after 9 days. Similar behavior was observed for *Ips ponderosae*, although this species continues to enlarge the nuptial chamber more actively by attaching galleries in one or more directions (Fig. 6).

Coinciding with the progress of the nuptial chamber or initial gallery, logs infested with *Ips confusus* and *Ips ponderosae*, as well as with *Dendroctonus brevicomis*, produced an attraction after 24 hours comparable to those infested for several days. Depending on the prevailing temperature such infested logs continued to be attractive for three to four weeks

(*Dendroctonus brevicomis*) during spring or two weeks during summer as observed for *Ips confusus* (Fig. 7). With the progress of larval galleries and the drying out of the sapwood, the attraction ceased rapidly as the new generation approached emergence.

Concentration and intensification of attractants. Under natural conditions, the number of beetles attacking a tree is limited. For *Ips confusus* a heavy attack generally does not exceed 20 to 25 entrance holes per square foot of bark; a heavy attack of *Dendroctonus brevicomis* seldom exceeds 12 to 16 entrance holes per square foot (15).

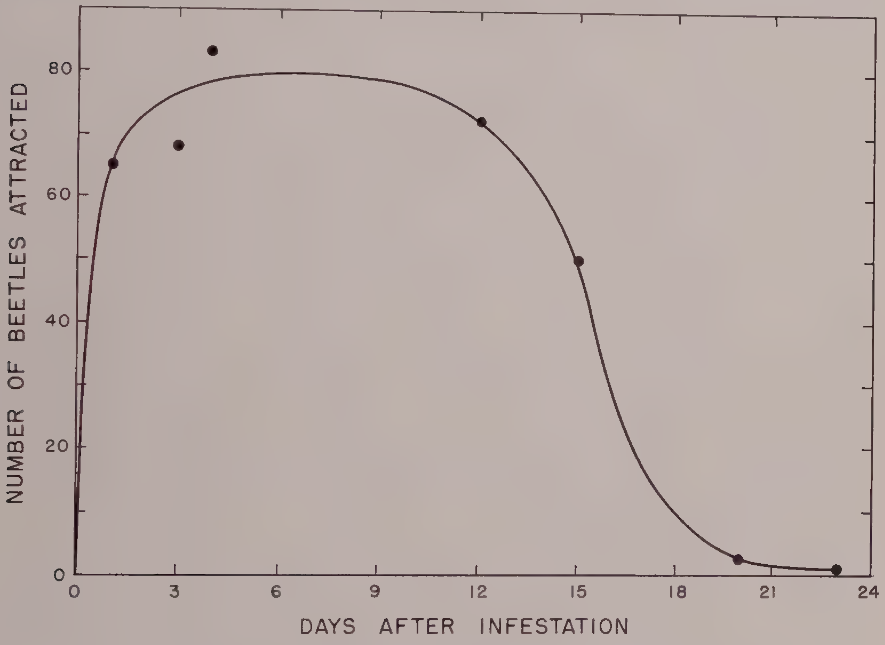

FIGURE 7. Attractiveness of log sections infested with *Ips confusus* in relation to the age of infestation, June 26 to July 26, 1961.

To determine whether the density of inoculation influences the attractive power of artificially infested logs, 25 male beetles of *Ips confusus* were implanted into log sections with ½, 1, and 2 square feet of bark surface and subjected to the usual 24-hour test in olfactometers. These logs had been cut from the same tree. The results were uniform and did not indicate any differences in attractive power (Fig. 5).

In further experiments, an attempt was made to intensify the attractive power of infested logs artificially. Accidentally it had been observed that logs treated with stove oil and cycloheximide exhibited an obviously higher attraction than untreated logs. A series of tests using log sections treated with stove oil, diesel oil, and gasoline and subsequently infested with 25 male beetles of *Ips confusus* showed that stove oil and gasoline produced a higher attractive power (Table VII).

Distance of attraction. From experimentation on the relative attractive power of various amounts of infested material it was suggested that the number of attacks used in attraction influenced the distance over which bark beetles were drawn. Further evidence of this phenomenon was obtained during the incidental use of larger amounts of material infested with *Ips confusus* in two locations approximately 500 meters apart, both situated in the same stand of second growth ponderosa pine. When larger amounts of attractive material were used in one station than in the other, the number of beetles caught dropped markedly at the station with the weaker attraction.

TABLE VII

NUMBER OF IPS CONFUSUS ATTRACTED TO LOGS WITH VARIOUS TREATMENTS DURING A 10-HOUR TEST

Log section infested with 25♂♂ after treatment with	Number of beetles caught
Diesel oil	4
Diesel oil	5
Stove oil	86
Stove oil	90
Control	74
Control	76
Gasoline	317
Stove oil	210
Control	171
Gasoline	304
Gasoline	269
Gasoline	168
Control	153
Control	145
Control	115

Further experiments were concerned with the possible influence of wind on the distance of attraction and the response of bark beetle population to centers of attraction. For this purpose, six olfactometers were placed at equal distances along the circumference of a circle with a radius of about 500 meters throughout a ponderosa pine stand. The center was used as the release station of 1,430 beetles of *Ips confusus* marked with fluorescent dye. To insure prompt take-off, the marked beetles were exposed to direct sunlight for a short period and then lifted on a large blanket into the air by gently swinging the blanket. Two minutes after take-off of the first beetles, the remaining portion of the marked population was recollected from the blanket. In this experiment 157 marked beetles or 11 per cent of the released population were recaptured in olfactometers within two hours. With winds prevailing from the west, there was a preference in response to the olfactometer situated west of the release point. However, in this experiment as well as in several repetitions, the marked population also responded to olfactometers in all other directions (Table VIII). The frequent shifting of low velocity winds as well as the flight habit of bark beetles to disperse after release in all directions, but preferably with the wind, may explain this observation. An experiment in an opposite arrangement with six release stations in a circle around an attraction center revealed similar relations although conducted under slightly higher wind velocities; the *Ips confusus* population responded best against the prevailing wind direction, but also with the wind. None of the beetles released at the other stations which were outside the main wind direction were recovered.

TABLE VIII

NUMBER, LOCATION, AND TIME OF THE RECOVERY OF MARKED IPS CONFUSUS RELEASED AT THE CENTER OF A RING OF 500 METERS RADIUS

Marked beetles recovered		Location of olfactometer to point of release	Peak (and period) of time of recovery in minutes after release
No.	Per cent		
19	12	E	30 (20–50)
29	19	SE	30 (25–60)
22	14	S	30 (25–60)
46	29	W	30 (25–70)
24	15	N	30 (20–80)
17	11	NE	30 (25–60)

Although appreciable percentages of the marked beetles were attracted and recovered in all of these experiments, there were too few for a clear interpretation of the results. To investigate whether the marked populations responded to the olfactometers in a directed flight over large distances or in a random flight over shorter distances, the time of response was closely observed in these and further experiments. It was assumed that, using various distances between the point of release and the point of attraction, the time of response would show a linear relationship to various distances as long as directed flight prevailed. In contrast, attraction over short distances should have been marked by a decrease in percentage of recovery as well as by a nonlinear time increase in responding over increased distances between the point of release and the point of attraction. The results of this study are compiled in Table IX. Although conducted under various weather conditions, the percentage of recovered beetles is rather similar regardless of the distance, except where the marked population was released in the immediate vicinity of the olfactometer.

TABLE IX

NUMBER AND TIME OF RECOVERY OF MARKED IPS CONFUSUS RELEASED OVER VARIOUS DISTANCES AGAINST THE MAIN WIND DIRECTION

Total No. of beetles	Marked beetles recovered		Distance in meters and direction of point of release from point of attraction	Peak (and period) of recovery of marked beetles in minutes after release	Dominating wind speed, m.p.h., and direction
	No.	Per cent			
440	129	29	10 N	2 (0–20)	2–4 S
315	18	6	100 W	6 (4–36)	0–2 Changing
427	31	7	200 W	14 (8–40)	0–2 Changing
150	8	5	500 NW	40 (30–80)	3–6 SE
100	4	4	600 NE	50 (30–80)	3–6 SE

CONCLUSIONS

The experiments confirm the conception that bark beetles attacking ponderosa pine, their coleopterous predators, and other subcortical feeding insects concentrate on suitable host material through olfactory responses to volatile materials. These volatile materials are transferred by air movements and can be responded to over great distances. Experimentally, the attractants can be translocated by an air stream or with compressed air through pipelines. The fact that the bark beetle species investigated responded to artificial trapping devices which were fed with the attractant through plastic tubing of up to a length of 80 meters proves the predominance of the olfactory sense in orientation. It disproves the suggestion that other factors such as auditory or visual stimuli may be responsible for the mass attack of individual trees.

The nature and origin of the attractants, however, vary widely with species and ecological group. Although the studies reported are restricted to ponderosa pine, it becomes evident that the attractants may span from nonspecific products of fermentation processes in the phloem tissue, over volatile materials emanating from intact trees such as oleoresin vapors to highly specific sex attractants or possibly the combinations of several compounds.

Fermentation products, as the least specific material characteristic for the condition of the host material, seemed to attract only saprophagous insects (certain Bostrychidae). Raw oleoresin attracted secondary bark beetles such as *Dendroctonus valens* and several *Hylastes* spp. while the scolytid species capable of primary attack responded to species specific attractants only.

Of major interest in this study was the source of the materials attractive to primary attacking bark beetles such as *Dendroctonus brevicomis* and *Ips confusus*. According to leading theories (4, 15) their attractants were believed to originate from yeasts. As a result of this study, however, microorganisms associated with *Dendroctonus brevicomis* and *Ips* species might be ruled out as a possible source of attraction for several reasons. The attraction is produced spontaneously within 24 hours and ceases at a time before yeasts and other microorganisms can reach optimal development, or the deterioration of the phloem by such organisms can occur. In addition, the attractants are species specific, but the association between bark beetles and yeasts is not (22). Furthermore, the impregnation of log sections with chemicals toxic to yeasts did not reduce their attractiveness after infestation with beetles, and logs from which the bettles were removed lost their attraction. Other objections have been made recently: the weak capacity of the yeasts involved in the fermentation of monosaccharides (4, 11, 22) and the failure of either male or female beetles and premature beetles to produce attractants (27).

From the experiments conducted it can be concluded that the production of species specific attractants is limited to one sex. In the polygamous genus of *Ips* this capacity is limited to the mature male beetles; in the monogamous species, *Dendroctonus brevicomis* and *Dendroctonus monticolae*, the attractant is produced by the mature female. As an ecological convergence, in both genera the sex initiating the attack on new host material, and thus responsible for the host selection, is capable of producing the attraction for mass attack.

Very obviously, the production of the attractants depends upon contact with suitable breeding material. Mature beetles outside of fresh phloem or in dry phloem or crushed beetles homogenized with phloem do not create attraction. There are two possible explanations or their combination for this phenomenon. The production of the attractant is stimulated by the contact with suitable host material or the volatilization and emanation of the attractants may possibly depend on other volatile materials such as oleoresin emanating from the xylem. In fact, two of the experiments indicated such relationships. Logs from which all beetles and the bark were removed continued to exhibit some attractiveness due to the exposed xylem portions of the galleries, and the treatment of infested log sections with volatile solvents such as gasoline and kerosene increased the attraction.

Of importance for an application in bark beetle control are the questions whether it is possible to compete with naturally occurring sources of attractants such as recently attacked trees, and secondly, over what distances baited traps would be effective. Even though these studies provide some useful indications, much more information on flight behavior and pattern of host selection of the bark beetle populations is needed to thoroughly evaluate these problems.

Under natural conditions, the attraction of a tree increases with the number of attacks; however, the numbers of attacks occurring on a tree are limited by the bark surface available. In contrast, the number of beetles can be multiplied by the artificial infestation of small log sections. Besides this concentration, the attraction can be intensified by air movement or by applying volatile solvents like gasoline or kerosene. With such treatment, small log sections with a bark surface of 225 $cm.^2$ infested with 25 male beetles of *Ips confusus* became an attraction equal to a medium size trap tree.

An increased intensity of attractants, in turn, seems to result in increased attractive power over longer distances. Under endemic conditions where no or only weak natural sources of attraction competed with experimentation, marked beetles were obviously attracted over distances exceeding 500 meters. It is questionable, however, if such distances apply under epidemic conditions.

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STUDIES ON THE FLIGHT PATTERNS OF BARK BEETLES (COLEOPTERA: SCOLYTIDAE) IN SECOND GROWTH PONDEROSA PINE FORESTS[1,2]

R. I. Gara[3] and J. P. Vité[4]

SUMMARY

A study of the seasonal, diurnal, and local flight patterns of bark beetles was performed during the spring of 1961 in second growth stands of ponderosa pine with endemic and epidemic populations of *Dendroctonus brevicomis* Lec. and *Ips confusus* (Lec.). Rotary net traps were used for random sampling of bark beetles in flight and funnel-shaped bait traps (field olfactometers) for selective trapping of bark beetles responding to suitable host material. The patterns of emergence were observed by means of caged logs.

Seasonal and diurnal patterns of flight, represented by occurrence, although varying according to species, are shown to be dependent on weather conditions, particularly temperature. Although influenced by light, temperature, and wind, in accordance with the seasonal and diurnal patterns, the local flight patterns varied with population level and availability of sources of attraction.

From the data obtained it is concluded that the flight of *Dendroctonus brevicomis* and *Ips confusus* consists of two phases, dispersal and concentration. Both phases apply to the flight pattern of individuals as well as populations and can be interpreted as necessary steps in allocation and colonization of suitable breeding material. While temperature and light seem to influence navigation during the dispersal phase of flight, olfactory attraction became the dominating factor in concentration flights. Long periods of dispersal with concentration flights over long distances seem to prevail under endemic conditions, while short periods of dispersal followed by concentration over short distances may be more characteristic for epidemic conditions.

INTRODUCTION

Bark beetles complete their whole life cycle inside the host plant with the exception of short flight periods. During these flights, new hosts have to be found and colonized before broods can be successfully established. Thus, the bark beetle flights often consist of two phases: first, a dispersal flight in search of suitable breeding material and secondly, a concentration flight leading to colonization of such material. This is especially true for bark beetles capable of primary attack which depend upon the live tree for

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[2] Based on studies for a thesis to be submitted by R. I. Gara to the Graduate School of the Oregon State University in partial fulfillment of the requirements for a Master of Science degree.

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brood establishment. Here, both phases, dispersal and concentration, become the basic impetus of host selection.

In other words, host selection largely depends on the flight behavior and flight capacity of a species and, accordingly, navigation and flight activity determine where and when new hosts become selected by a bark beetle population. Thus, flight behavior and flight capacity lead to flight patterns which in turn result in patterns of host selection. The study of flight patterns, therefore, becomes an important aspect of bark beetle research in view of experimentation as well as control.

Investigations of flight patterns, however, present a major technical difficulty; the ocular observation of such small insects as bark beetles is not possible for distances greater than 100 feet (12, p. 47). The possibility of supplementing the lack of information on flight behavior of populations with observations resulting from laboratory studies on the responses of individual beetles to various flight stimuli seems to be rather limited. Also, most if not all experiments dealing with the flights of marked beetles failed or did not meet the expectations of investigators (8, 12)[5,6]. Similarly, observations on bark beetles emerging from caged logs or attacking trap trees have no direct bearing on the dispersal of the emerging population and the origin of the attacking population. Thus, conclusive data pertaining to dispersal and concentration of bark beetle populations are rare.

A direct approach in studying the flight pattern through trapping the insects in flight would be more promising provided that appropriate techniques can be developed. Such an approach was found in the study of Chapman and Kinghorn (4, 5) on the flight activities of *Trypodendron lineatum* (Oliv.) and other scolytids by means of flight traps such as window traps and rotary net traps. These studies, for the first time, quantitatively expressed occurrences of bark beetle flight in terms of seasonal rhythms as influenced by physical and biotic factors.

Essentially, there are two types of trapping techniques, random and selective. Random trapping is accomplished by devices that trap insects without use of attracting stimuli, and thereby capture by chance encounter. Selective trapping techniques capture insects by use of directing or luring stimuli causing insects to actually direct their flight to the trap. As expressed by Johnson and Taylor (11), trapping at random techniques are useful only when closely estimating the actual insect density in a given air volume. Therefore, among the random trapping techniques such as suction traps (11), moving nets (2), or the various stationary traps (4, 13)

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only those fulfill this requirement that actively engage in trapping while still sampling a predetermined and constant air volume. Such devices include the suction and rotary net traps.

In contrast, selective trapping techniques utilize the fact that some insects are attracted by certain stimuli such as odors, light, colors, etc., which are incorporated in the trapping devices (7). Even though it is generally believed that response to olfactory stimulation is the predominant factor in host selection for many Scolytidae, little attention has been given to the development of actual trapping devices that incorporate this phenomenon. As an exception, Chararas (6) recently reported limited experimentation with small funnel-shaped trapping devices hung on the branches of trees, and baited with various compounds contained in oleoresin. In general, the use of bait traps in bark beetle research was previously limited to the use of trap trees (12). The difficulties encountered by this method, mainly the tedious detection of attacking beetles and the differences in attractiveness of individual trap trees, were partially overcome through the use of insecticides. Logs treated with a residual spray and elevated above a collection tray served for observation of flight occurrences and the relative densities of attracted populations (3, 5). Such trapping devices may suffice to establish flight patterns of some bark beetles which are attracted by the host material *per se*. This method, however, is of rather limited value when used with bark beetles whose host selection depends on species specific sex attractants (17).

The study described here is an effort to investigate the flight pattern of scolytid populations in stands of second growth ponderosa pine, *Pinus ponderosa* Laws. By applying random and selective sampling methods under both endemic and epidemic population levels, it was hoped that an insight would be obtained into some of the factors governing the various phases of bark beetle flight and host selection.

MATERIALS AND METHODS

Two pieces of apparatus were used throughout this study: rotary net traps for random sampling and field olfactometers baited with freshly infested log sections of ponderosa pine for selective sampling. Both were standardized as described elsewhere (16, 17). The rotary net trap assembly consisted of a single deep nylon net 38 cm. in diameter and 70 cm. deep which was affixed to the center of rotation by a radial arm 235 cm. in length. The center of rotation was connected, by means of a shaft-coupler, to a $\frac{1}{4}$ horse power electric motor of 60 r.p.m. which enabled the net to travel at a speed of 53 km./hr., while sampling air at the rate of 6,000 cu. meters per hour. The entire assembly was supported on 2-meter aluminum ladders. Thus, the rotary net traps could be moved easily from one sampling area to another.

The field olfactometers used in these experiments were basically sheet metal tubes that fitted over infested logs known to be highly attractive. Air was drawn over the logs by a fan and exhausted through a funnel. The funnel contained an inverted wire-mesh cone which terminated in a removable collection bottle. Glass plates set across the mouth of the funnel served as a colliding barrier to the insects attracted by the device. In contrast to trap trees and other devices, the insects caught in the device were readily available for immediate evaluation. Furthermore, the attractive log sections used in the field olfactometer maintained a rather uniform attraction power for extensive time periods as the log sections were prevented from additional attacks by the field population (17).

This equipment was set up in various locations in stands of second growth ponderosa pine; the equipment operated either from power line sources or from a portable generator. Checks were usually made at one- or two-hour intervals and the material caught was collected for examination. In addition, ponderosa pine logs naturally infested by bark beetles were taken from representative locations and caged in an outside insectary in order to observe the emergence pattern. Temperature and humidity were recorded continuously, while other weather information such as wind velocity and direction were taken periodically.

The studies were carried out from February 20 to July 20, 1961 in the BTI experimental forest near Grass Valley, California which is rather uniformly stocked with 60- to 95-year-old ponderosa pine growing at elevations of from 2300 to 2500 feet. Power lines throughout the forest facilitated operation of the equipment. A comparable study was performed under epidemic conditions in an area previously described (18) near Crystal Bay, Nevada.

RESULTS

The data obtained indicated that bark beetle flights exhibit definite seasonal, diurnal, and local patterns. These patterns were mainly influenced by sources of attractants and weather conditions. Temperature seemed to be the most limiting factor.

Seasonal flight pattern. The first flight of the season occurred in the Grass Valley area in late February when average daily temperatures reached 16° C. During this initial flight the rotary nets caught 10 scolytids per hour with *Phleosinus* spp. predominating. The ensuing flights were extremely temperature dependent (Fig. 1), occuring only above 15° C. However, later in the season when temperatures above 20° C. prevailed, the flights sometimes became less dense. The most dense flights of the entire spring season occurred in late March when the rotary nets recorded catches of 16 scolytids per hour.

The seasonal flights in Grass Valley initially began with dense *Phleo-*

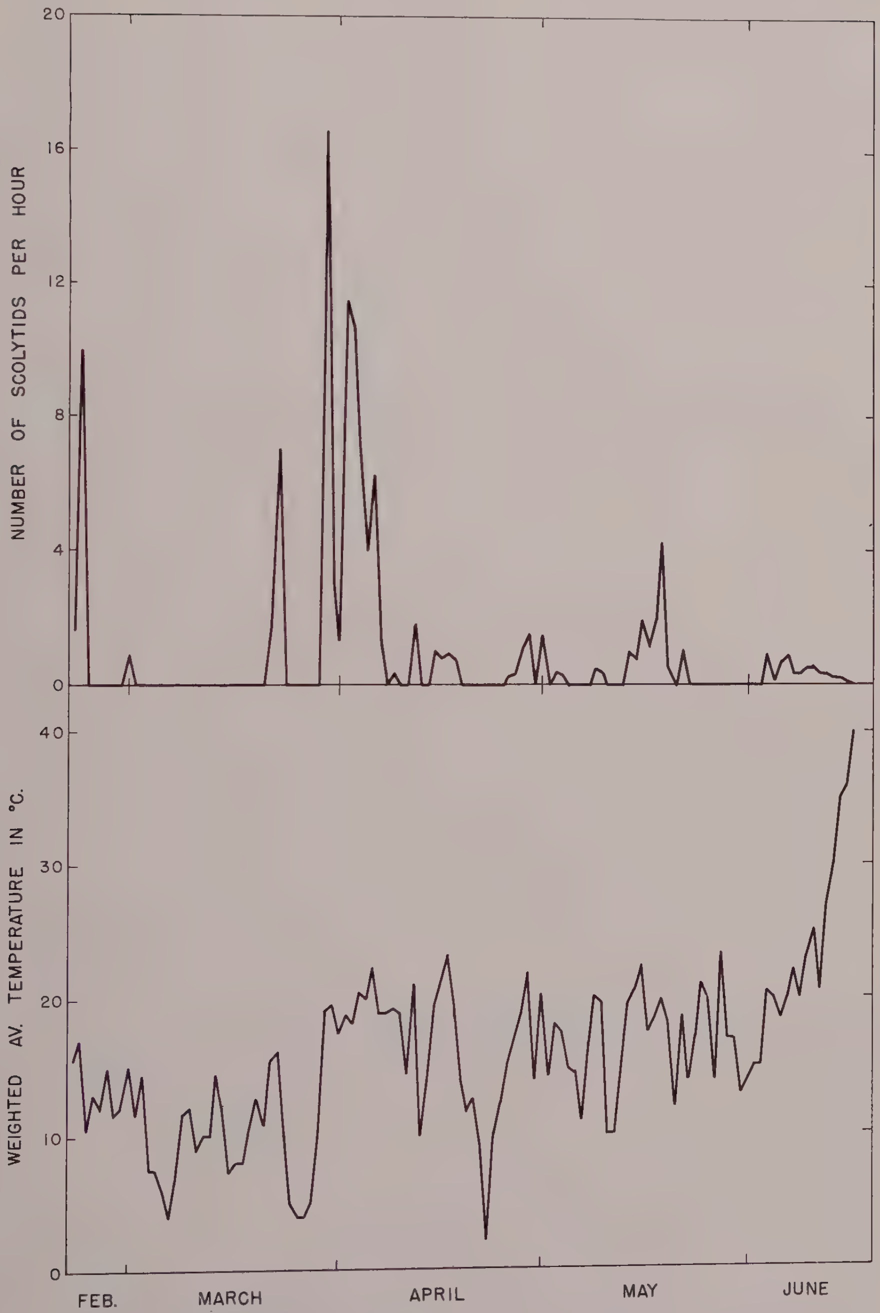

FIGURE 1. Seasonal flight patterns of scolytids in relation to weighted average temperature as obtained at site of the operation of rotary net traps in Grass Valley.

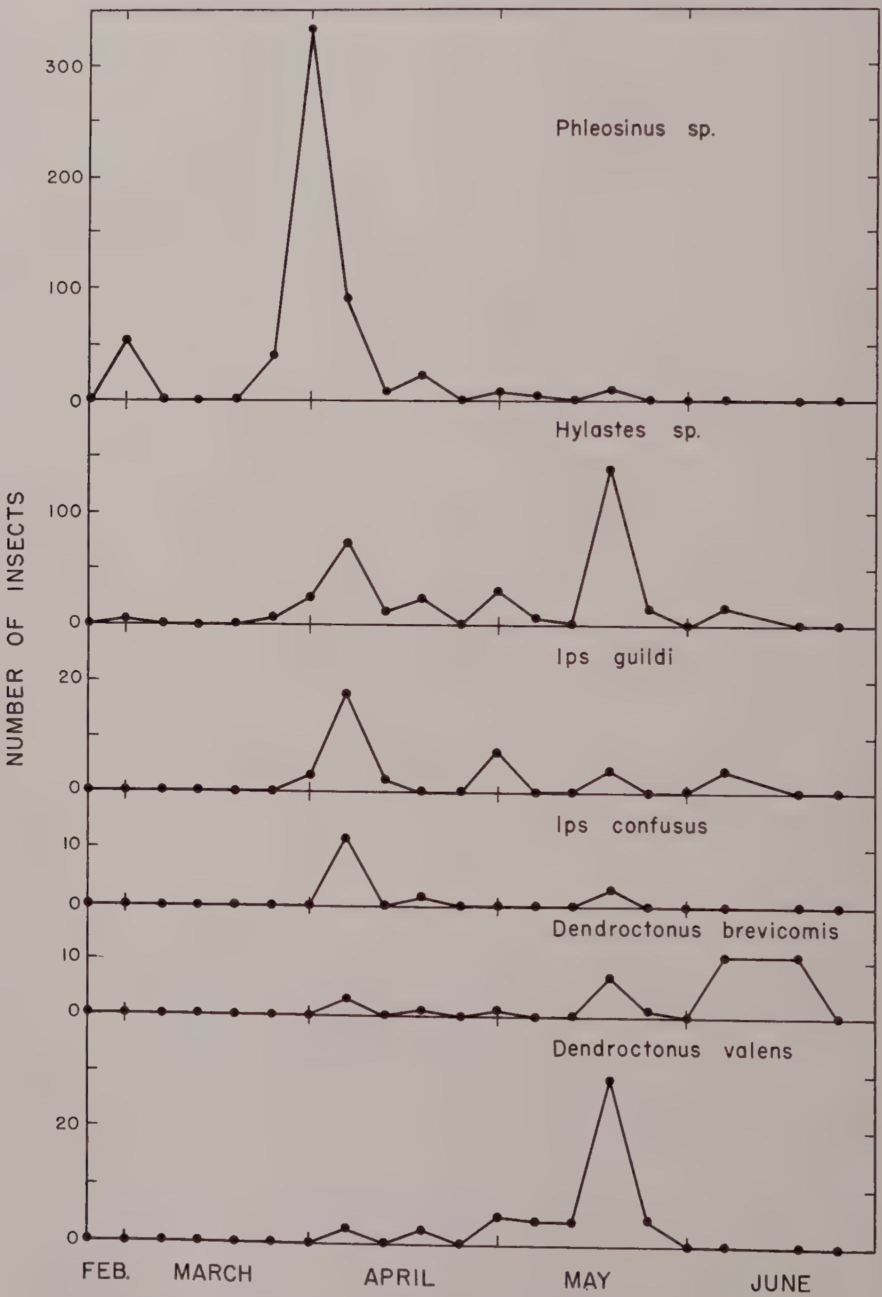

FIGURE 2. Seasonal flight patterns of various scolytids as obtained from rotary net traps operating in Grass Valley.

sinus spp. flights and to a lesser extent with various *Hylastes* species. *Phleosinus* had two major flights (Fig. 2) in February and March with a final decline in mid May, while *Hylastes* had peak flights in late March and mid May with terminating flights in early June. Also appearing in early April were peak flights of the overwintering generations of *Ips guildi* (Blkm.) and *Ips confusus* (Fig. 2). The second generation of *Ips confusus* emerged and flew in mid May, while *Ips guildi* continued with three additional low density flights through the first part of June.

No major *Dendroctonus* catches were made until early April when overwintering adults of both *Dendroctonus brevicomis* Lec. and *Dendroctonus valens* Lec. appeared in low densities (Fig. 2). The flight density of *Dendroctonus valens* increased in early May to a maximum in mid May. After a sudden decline this species continued to appear regularly in small numbers throughout the season. The population of *Dendroctonus brevicomis* which hibernated as larvae emerged and flew in mid May but continued to occur in higher densities through early June.

In addition to the much higher elevation, unusually cool weather throughout the spring in the Crystal Bay area delayed the flight of *Dendroctonus brevicomis* until June. Here, the flight pattern was initially coincidental with the falling and rising temperatures. However, this trend became erratic in mid June as temperatures rose above 23° C.

The pattern of random flight, of emergence and response to field olfactometers, was investigated more closely for *Dendroctonus brevicomis* in both areas. Emergence and random flight coincided rather closely under both epidemic and endemic conditions. The response of the population, however, to sources of attractants seemed to be retarded for one or two days in the Grass Valley area where endemic conditions prevailed. Under the epidemic conditions at Crystal Bay the population responded less distinctly to the olfactometers but peaks of emergence flight and response occurred usually on the same date (Fig. 3).

Diurnal flight pattern. As was expected, the diurnal flight pattern varied with the species and changed somewhat with the progressing season as a result of changing temperature and light conditions. The main flight activity of all scolytids observed occurred during the afternoon and evening. *Dendroctonus valens*, *Ips confusus*, and *Phleosinus* flew relatively early in the afternoon with peaks around 1:00 P.M. in the spring (Fig. 4) and 4:00 to 6:00 P.M. during summer while *Dendroctonus brevicomis* was found in densest flight between 4:00 and 6:00 P.M. (Fig. 5). *Hylastes* spp. [i.e., *Hylastes macer* Lec., *H. nigrinus* (Mann.), and *H. minutus* Blkm.] preferred the evening hours with peaks consistently at 6:00 P.M. during spring and summer in the Grass Valley area as well as in the Crystal Bay area. The consistent peak flight time suggests a circadian rhythm (10) rather than a temperature dependent diurnal flight pattern (Fig. 4).

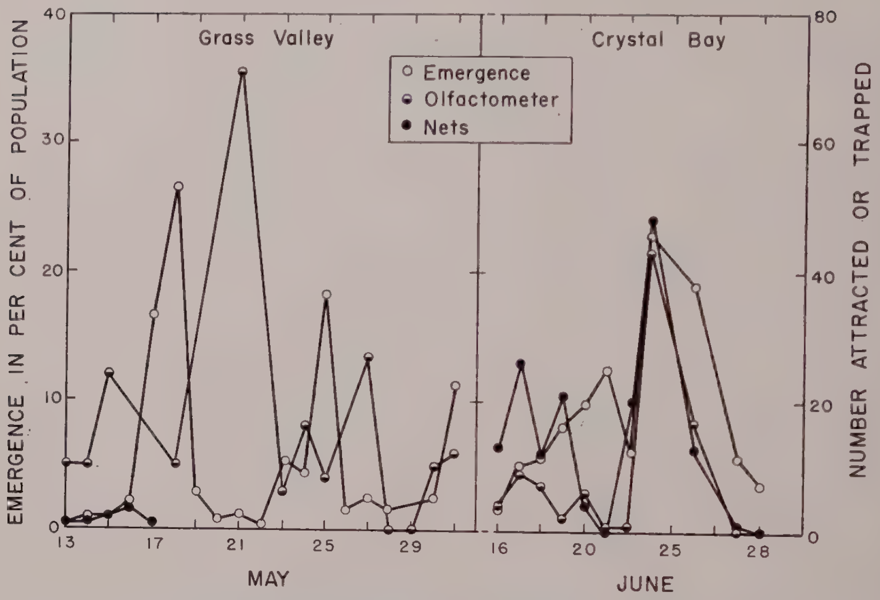

FIGURE 3. Seasonal emergence (left hand scale), flight, and response pattern (right hand scale) of *Dendroctonus brevicomis* as recorded in Grass Valley and Crystal Bay.

In contrast to emergence and random flight, the response of several scolytids to olfactometers showed two peaks during the late spring and summer, one in the morning and one in the late afternoon or evening.

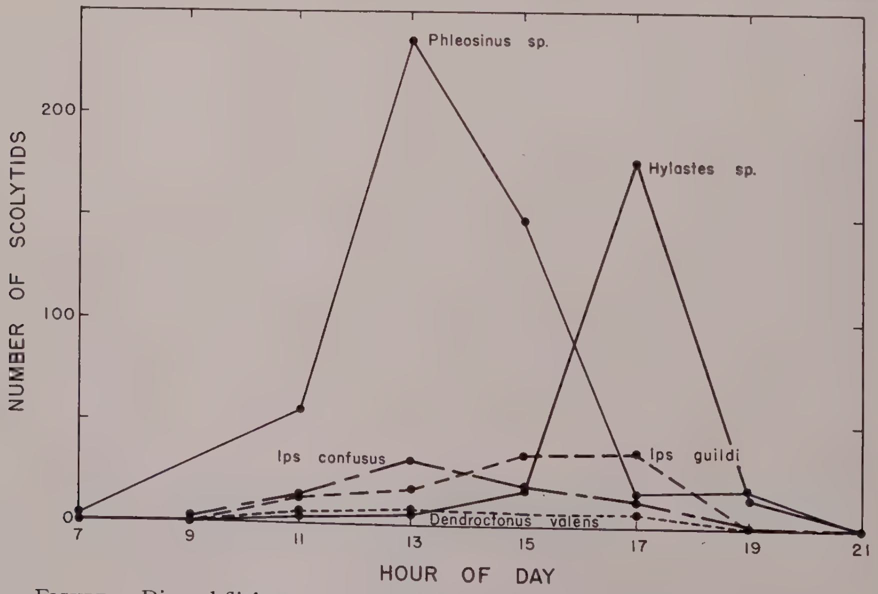

FIGURE 4. Diurnal flight pattern of various scolytids as obtained from rotary net traps operating in Grass Valley during February to April.

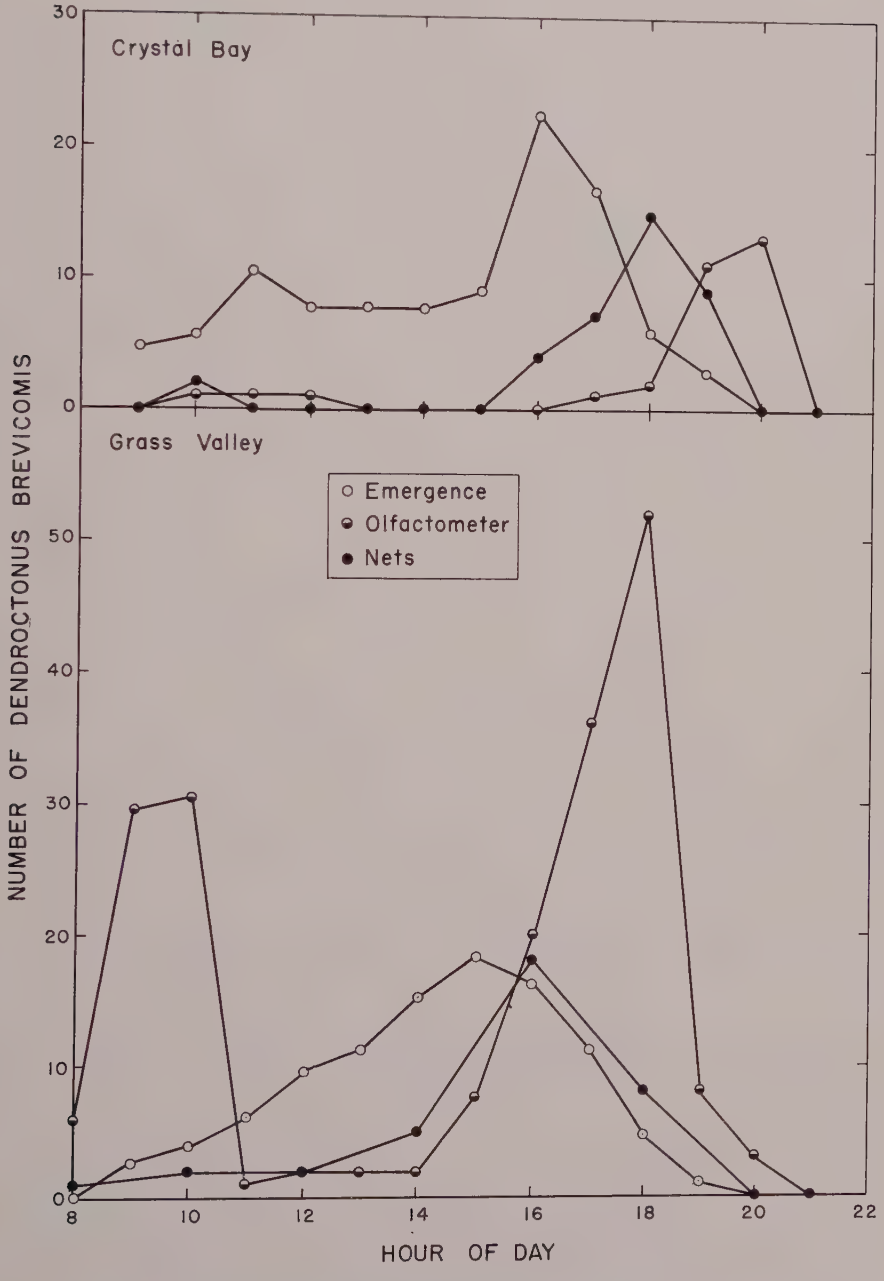

FIGURE 5. Diurnal emergence, flight, and response patterns of *Dendroctonus brevicomis* in Grass Valley and Crystal Bay.

As shown for *Dendroctonus brevicomis* in Figure 5, the peaks of emergence, random flight, and response to the olfactometers followed one another in one- or two-hour sequences. Without appreciable emergence or random flight, another distinct response peak occurred on the following morning. Again, temperature seemed to be the limiting factor responsible for this pattern. *Ips confusus* initiated flight in the morning at 19° C. but interrupted its response to olfactometers as soon as temperature rose above 28° C. (Fig. 6) while its predator, *Temnochila virescens* var. *chlorida* (Mann.), began the flight at 24° C. and continued to fly as long as temperatures did not rise above 34° C.

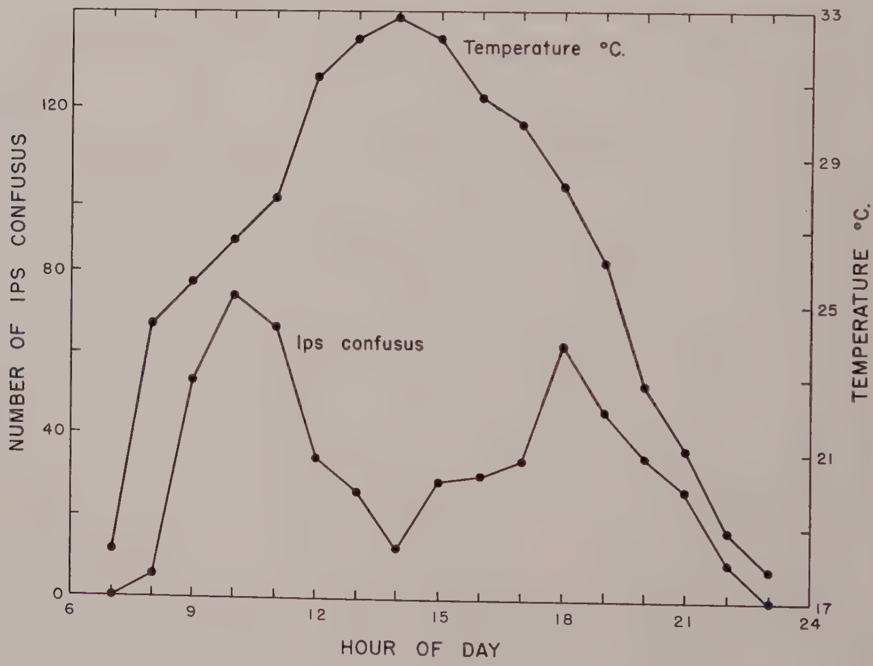

FIGURE 6. Diurnal response pattern of *Ips confusus* in relation to temperatures in Grass Valley.

Essentially, as observed for *Dendroctonus pseudotsugae* Hopk. (1, 14), the upper limiting temperatures for flight were between 33° and 35° C. However, with air temperature well below 33° C. the beetles may cease to fly. This reaction was probably due to their body temperatures which must have been considerably higher than the surrounding air as a result of heat absorption through solar radiation. To determine the possible magnitude of heat absorption, thermometers were inserted in a narrow vial and placed in the sun. After the temperature was recorded, the measurement was repeated with *D. brevicomis* completely covering the mercury bulb. As expected, the black insect bodies absorbed heat producing a higher thermometer reading. The experiment was again performed with the beetle covering the bulb, but in a stream of air produced by a small fan, thus simulating the beetle's body temperature while flying. This final experiment also indicated that even a flying insect can absorb enough solar heat to raise the body temperature five or more degrees.

Local flight pattern. Naturally, the flight density observed with random trapping devices in various locations depended on the local population level. In the Crystal Bay area, six times more *Dendroctonus brevicomis* were caught than in a comparable period in Grass Valley (Fig. 7). In contrast, the olfactometers caught less *Dendroctonus brevicomis* per time unit under the epidemic conditions than under the endemic population level at Grass Valley in spite of the much higher flight activity in the Crystal Bay area. The olfactometer could not compete with the many, naturally occurring sources of attraction.

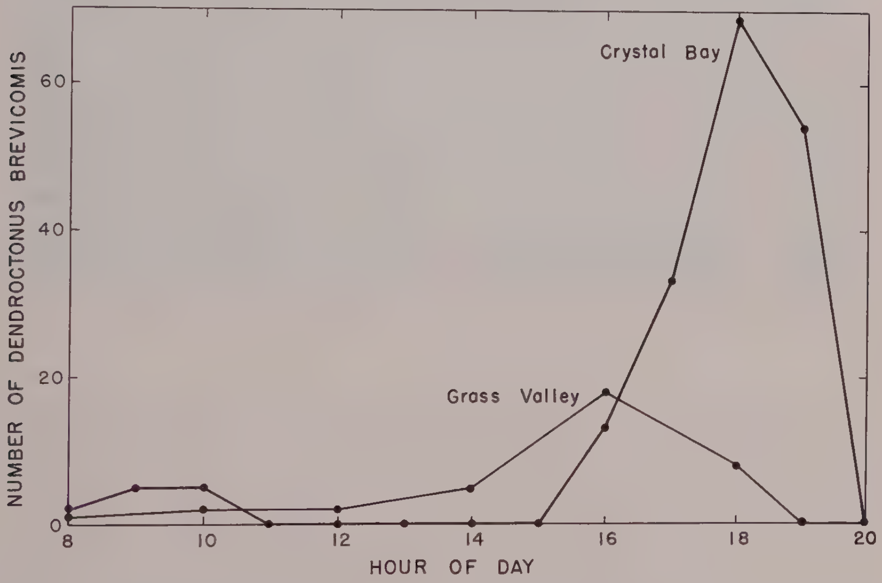

FIGURE 7. Comparative diurnal flight of *Dendroctonus brevicomis* in Crystal Bay and Grass Valley as obtained from rotary net traps operating during a comparable period.

However, there were many indications that the scolytid populations preferred certain localities within each area. Thus, aerial net traps in various positions in the Crystal Bay area showed the preference of the beetles to shaded places, while in small openings considerably fewer beetles were caught during the same time interval. Olfactometer tests in Grass Valley confirmed these observations. Olfactometer catches in the shade of trees were consistently higher than on sun-exposed openings and, interestingly

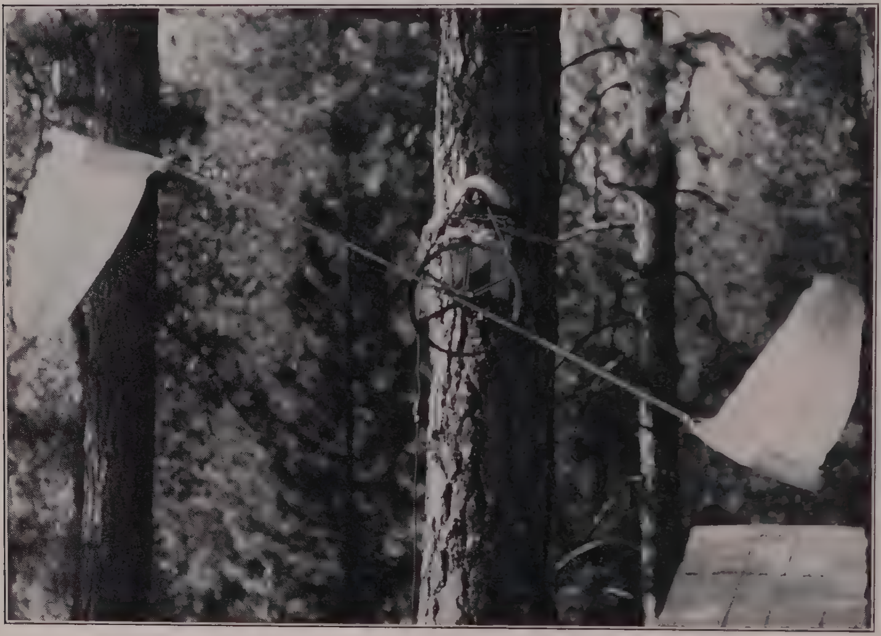

FIGURE 8. Vertically operating rotary net trap fastened to a tree.

enough, the highest number of insects were caught just inside the stands at the borderline of openings. However, rotary nets affixed to the main bole of trees in a vertical position (Fig. 8) caught consistently fewer Scolytidae but more Buprestidae and Cerambycidae than nets operating in a horizontal position a few meters away from the trunk.

Rotary nets in various vertical positions (Figs. 8 and 9) did not reveal differences in flight activity from the ground to the lower crown region. In the upper crown region and above forest stands, however, bark beetles seemed to fly less frequently.

DISCUSSION

The observations presented confirm the well known influence light and temperature have as initiating and limiting factors on flight activity and as orienting factors in navigation of bark beetle flights. They determine the flight periods, dominate the diurnal flight pattern to a large degree and influence the local flight pattern at least to some extent. Together with all other orienting factors, the influence of light and temperature on navigation, however, seems to become limited as soon as attraction is encountered. *Dendroctonus brevicomis* and *Ips confusus* were found to respond to field olfactometers in openings with high temperature and light intensity where flight normally did not occur. In addition, responding beetles continued to

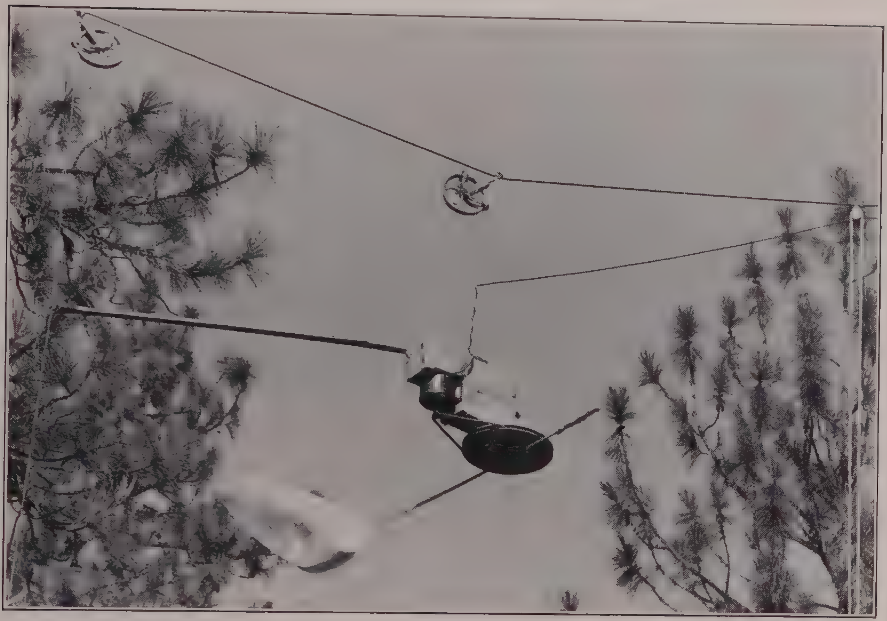

FIGURE 9. Rotary net trap assembly fastened to a hoist for sampling air spaces at various heights.

crawl on the outside of the olfactometers, even though the heat, radiating from the sun-exposed metal became fatal within a few minutes. Attraction, where and when it exists, does not alter light-temperature dependent seasonal and diurnal flight patterns but dominates the local flight pattern. These observations suggest the existence of two distinct phases in the flight period of an individual as well as a population which may be designated as dispersal flight and concentration flight.

Evidently, under endemic conditions where suitable hosts are scarce, the individual flights extend over a much longer period of time than under epidemic conditions where sources of attraction occur sooner and more frequently. Observations at Grass Valley indicate that for *Dendroctonus brevicomis* the peak of response lagged two days behind peak emergence, and the diurnal pattern exhibited morning responses of large numbers of beetles which emerged on previous days. With the flight period progressing into the concentration phase, peaks of emergence and response coincided. In contrast, emergence, random flight, and response coincided in the Crystal Bay area throughout the flight period without comparable figures in morning responses. There were further indications that under epidemic conditions short concentration flights prevail. The number of *Dendroctonus brevicomis* caught in olfactometers was higher under endemic conditions than under the epidemic conditions at Crystal Bay although the population was much lower. Most likely, beetles must have been attracted over considerably longer distances in the endemic area whereas at Crystal Bay beetles responded over short distances. This is also supported by a comparison between the number of *Dendroctonus brevicomis* caught in rotary net traps at Grass Valley and Crystal Bay. At Crystal Bay, the catches were only six times greater than during a comparable time period in the endemic area. Naturally, the population at Crystal Bay must have been considerably more than six times that of Grass Valley which can be assumed from the number of existing brood trees at the rate of 0.05 per cent at Grass Valley and 3 to 5 per cent at Crystal Bay (18). *Dendroctonus brevicomis* and likely *Ips confusus* do respond to sources of attraction soon after emergence from their brood tree. Flight exercise does not seem necessary to make the insect responsible to olfactory stimuli as suggested for other scolytids (9). In fact, responses to olfactory stimuli seem to overpower responses to any other stimulus to a degree rarely found in the host selection behavior of other groups of insects (15).

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BIOCHEMICAL AND PHYSIOLOGICAL STUDIES OF TISSUE CULTURES AND THE PLANT PARTS FROM WHICH THEY WERE DERIVED. II. GINKGO BILOBA L.[1]

Walter Tulecke, Leonard H. Weinstein, Alan Rutner, and Henry J. Laurencot, Jr.

SUMMARY

Free and protein amino acids, sugars, nonvolatile organic acids, and nucleic acid phosphorus of the pollen of *Ginkgo biloba* and a tissue culture derived from the pollen were determined. The concentrations of some constituents of leaf, nucellar tissue, and germinated pollen were also determined. The data indicate that the tissue culture resembled germinated pollen more than the other tissues analyzed. Citric acid was the main organic acid in both tissue and pollen. Glutamine and asparagine were predominant in the pollen and alanine and glutamine in the tissue. Sucrose was the major sugar in the pollen and glucose and fructose in the tissue. An unidentified neutral substance, possibly a glycoside or a naphthoquinone, was isolated from the pollen.

This is the second in a series of papers (26) on the comparative biochemistry of plant tissue cultures and the plant parts from which they were derived. These studies were initiated to determine the major differences and similarities between tissue cultures, which are characterized by a rapid indeterminate growth, and the analogous plant tissues, which have a specific function integrated with the whole plant. This paper presents data on the composition of a tissue culture from the pollen of *Ginkgo biloba* L. and ungerminated pollen. Both the tissue and the pollen were analyzed for free amino acids, protein amino acids, nonvolatile organic acids, sugars, and nucleic acid phosphorus. Some growth data for the tissue culture and some analyses of other plant parts of *Ginkgo* are included.

Ginkgo biloba L. is a broad-leaved gymnosperm indigenous to China, which is widely used as an ornamental tree. It is an important paleobotanical link between the ferns-cycads and the more recent gymnosperms. It is also known for its remarkable resistance to pests and disease (14).

During studies on the germination and viability of *Ginkgo* pollen, a tissue culture from the pollen was obtained (21). A culture of tissue (strain 5857) which grew well on media containing arginine (22) was used in the present study.

Ginkgo pollen tissue has a basic chromosome number of 12 (haploid) and also contains heteroploid cells. The tissue is white, undifferentiated, and is composed of meristematic cells which mature as storage parenchyma.

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[1] These investigations were aided, in part, by funds from the National Science Foundation grant No. G-17747.

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Liquid cultures of 16 liters capacity may produce several pounds of tissue in one batch run (23).

Changes in the biochemical composition of the medium and cells of a liquid culture of strain 5857 have been studied.[2] Arginine, sucrose, and citric acid accumulated in the tissue in the early stages of growth. Arginine utilization was greatest during the second week and γ-aminobutyric acid was present in both the tissue and the medium at this time, probably as a degradation product of arginine metabolism. With respect to sucrose uptake and hydrolysis, the tissue culture was found to behave very much like a group of pollen tubes. Sucrose was rapidly taken up and hydrolyzed.

MATERIALS AND METHODS

Samples of pollen were obtained by collecting strobili from male *Ginkgo* trees in early May. The fully mature strobili were spread out on paper and air dried to release the pollen. The latter was sifted through fine screens and frozen at −30° C. until used for analysis. A sample was collected from different trees in 1958, 1959, and 1960. The 1958 sample was stored for 6 months, the 1959 sample for 22 months, and the 1960 sample for 10 months before being analyzed.

The pollen tissue was grown in test tubes on a defined agar medium and was harvested for analysis after four weeks' growth. The growth response of the tissue culture to various sugars was calculated as the growth increment, i.e., the final fresh weight minus the initial fresh weight, divided by the initial fresh weight, multiplied by 100. The average inoculum size was 200 mg. per tube and five replicates were used.

Germinated pollen was obtained by taking strobili and surface sterilizing them in 20 per cent Clorox (1 per cent sodium hypochlorite) for 20 minutes, rinsing with sterile distilled water, and blotting with sterile paper towels. The strobili were then cultured on the same synthetic medium used for tissue growth (22). The germinated pollen burst from the pollen sacs as it grew and was harvested after 32 and 44 days' growth.

Mature leaf tissue was collected eight weeks after the buds opened in the spring. Twenty-gram samples were taken for analysis. The nucellar tissue, which serves as the natural source of pollen nutrient in the intact plant, was obtained from seeds at three and four months' post-pollination. The entire nucellus was peeled from the female gametophyte, weighed, and homogenized in 80 per cent ethanol.

The analytical techniques for free amino acids, protein amino acids, organic acids, sugars, and ribonucleic acid phosphorus (RNA-P) and deoxyribonucleic acid phosphorus (DNA-P), have been described previously (26). All tissue and pollen samples were prepared by homogenizing in 80 per cent ethanol and extracting five times at 50° C. The alcohol-insoluble residues were lyophilized and saved for analysis.

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[2] Tulecke, Walter, and Alan Rutner. Changes in the biochemical composition of medium and cells of a plant tissue culture during growth in a liquid medium containing arginine. (In manuscript.)

RESULTS

The free amino acid content of pollen collected in three different years is given in Table I. Glutamine and asparagine were the principal amides in all samples of pollen; consistently low levels of glycine, lysine, tyrosine, hydroxyproline, and methionine were observed. Arginine and aspartic and glutamic acids also were abundant in the 1958 sample. The variability of the individual amino acid content may be attributable to losses during storage, to the variation between individual trees, or to weather conditions. However, the over-all pattern of amino acids was fairly consistent and the total free amino acids did not vary greatly from year to year.

Data on the free amino acids of germinated pollen, pollen tissue culture, nucellus, and leaves of *Ginkgo* are given in Table II. Alanine and glutamine predominated in all tissues except in the nucellus where aspartic acid was high. Histidine, methionine, lysine, tyrosine, and hydroxyproline were absent or they were present only in trace amounts in all tissues. Appreci-

TABLE I

FREE AMINO ACIDS AND AMIDES OF GINKGO POLLEN

Amino acid	μG./100 mg. air-dried pollen*			
	1958	1959	1960	Average value
Cysteic	—	113	135	83
Aspartic	267	102	98	156
Glutamic	232	116	91	146
Serine	110	142	181	144
Glycine	—	14	17	10
Asparagine	284	341	463	363
Threonine	32	50	86	56
Alanine	76	78	103	86
Glutamine	877	825	702	801
Histidine	180	52	37	90
Lysine	33	—	4	12
Arginine	374	101	148	208
Proline	109	160	151	140
Valine	159	148	228	178
Leucines	114	121	193	143
Phenylalanine	40	35	77	51
Tyrosine	—	4	8	4
γ-Aminobutyric	43	54	115	71
Hydroxyproline	—	Trace	Trace	Trace
Methionine	—	29	14	14
Total	2,930	2,485	2,851	2,756

* Each year's results represent the average of two separate determinations.

TABLE II

FREE AMINO ACIDS AND AMIDES OF GERMINATED POLLEN, POLLEN TISSUE CULTURE, NUCELLUS, AND LEAVES OF GINKGO

Amino acid	μG./g. fresh weight			
	Germinated pollen (44 days' growth)	Tissue culture	Nucellus	Leaves
Cysteic	25	3	25	58
Aspartic	12	1	285	56
Glutamic	31	13	—	29
Serine	79	11	27	15
Glycine	30	3	16	9
Asparagine	39	Trace	Trace	—
Threonine	21	2	8	7
Alanine	468	74	90	77
Glutamine	156	28	38	24
Histidine	—	—	—	—
Lysine	—	3	—	—
Arginine	52	17	31	—
Proline	65	7	—	—
Valine	24	13	12	4
Leucines	29	13	36	—
Phenylalanine	—	4	—	10
Tyrosine	—	4	Trace	—
γ-Aminobutyric	39	18	33	5
Hydroxyproline	—	—	—	—
Methionine	Trace	—	—	—
Total	1,070	214	601	294

able amounts of asparagine were present only in germinated pollen. The leaves differed from other tissues in having small amounts of amides and basic amino acids, whereas the amounts of alanine, cysteic, aspartic, and glutamic acids and glutamine were high.

The nucellus was included for analysis because it served as the natural source of nutrients for the developing pollen tube in the young ovule. The nucellus contained alanine and glutamine, as did the other tissues analyzed, but, in addition, a high level of aspartic acid was found.

About three times as much protein amino acids was present in pollen as in the pollen tissue culture on a dry weight basis and eight times as much as in germinated pollen (Table III). The histidine, arginine, phenylalanine, and cysteic acid found in pollen protein disappeared after 44 days of germination and growth *in vitro*.

The composition of protein from the tissue culture resembled in some respects the germinated pollen and in other respects the ungerminated pollen (Table III). For example, the tissue culture and the germinated pollen contained no histidine, and arginine was present only in the tissue and ungerminated pollen.

The amounts of nonvolatile organic acids of the pollen tissue culture and leaves are summarized in Table IV. Citric acid was the main organic

TABLE III

Protein Amino Acids of Ginkgo Pollen, Germinated Pollen, and Tissue Culture

Amino acid	μG./100 mg. alcohol-insoluble residue			Per cent nitrogen of total amino acid nitrogen in protein hydrolyzate		
	Pollen	Germinated pollen*	Tissue culture	Pollen	Germinated pollen*	Tissue culture
Cysteic	53	—	837	0.1	—	5.7
Aspartic	2,616	319	167	7.3	8.3	1.4
Glutamic	2,389	460	994	6.0	10.9	7.8
Serine	1,404	197	225	4.9	6.5	2.5
Glycine	1,703	199	331	8.4	9.2	5.1
Threonine	1,145	131	293	3.6	3.8	2.9
Alanine	1,621	262	649	6.7	10.2	8.5
Histidine	1,395	—	—	10.0	—	—
Lysine	2,315	214	1,071	11.7	10.2	17.0
Arginine	1,980	—	711	17.0	—	18.9
Proline	251	330	232	0.8	10.0	2.3
Valine	1,720	217	700	5.4	6.4	6.9
Leucines	4,965	625	1,757	14.0	16.6	15.6
Phenylalanine	1,285	—	394	2.9	—	2.8
Tyrosine	356	68	265	0.7	1.3	1.7
Hydroxyproline	—	Trace	—	—	Trace	—
Methionine sulfoxide	198	225	109	0.5	6.6	0.9
Total	25,396	3,247	8,735			

* Pollen grown for 44 days on the same medium used for the tissue culture (22).

acid of the pollen and the tissue culture, and malic acid was present at a much lower concentration. Quinic acid was found in the tissue culture, but shikimic and pyrrolidone carboxylic acids were absent; all three organic acids were present in the pollen. Several acids which occurred in small

TABLE IV

Nonvolatile Organic Acids of Ginkgo Pollen, Pollen Tissue Culture, Nucellus, and Leaves

Organic acid	μEq./g. fresh weight			
	Air-dried pollen	Tissue culture	Nucellus	Leaves
Shikimic	7.3*	—	7.8	97.7
Quinic		Trace	11.1	84.6
PCA	14.7	—	—	—
Malic	18.3	1.5	18.0	—
Citric	135.5	7.7	3.0	21.0
Unidentified**	58.0	3.8	21.5	16.8
Total	233.8	13.0	61.4	220.1

PCA = pyrrolidone carboxylic acid.
* Contains small amount of PCA and unknown acid.
** Includes phosphate and several unknowns.

amounts in the pollen and tissue were not identified. The leaves contained large amounts of shikimic, quinic, and citric acids and only traces of other organic acids. The quinic acid in this case was obtained in crystalline form and identified by the infrared absorption spectrum. The nucellus contained malic, quinic, shikimic, and citric acids.

Only qualitative determinations of the organic acids of germinated pollen were run because of the small amount of material available. Two harvests of cultured pollen were analyzed: one at 32 days growth *in vitro* and one at 44 days. The former (2.0 grams) yielded 0.029 milliequivalent of acidity and the latter (2.5 grams) gave 0.027 milliequivalent. Malic, quinic, succinic, and fumaric acids were present in both samples. Pyrrolidone carboxylic acid and shikimic acid were not detected (as was found for the tissue culture).

TABLE V

SUGARS OF GINKGO POLLEN AND POLLEN TISSUE CULTURE

Sugars	Mg./g.		
	Pollen (dry wt.)	Tissue culture	
		(Dry wt.)	(Fresh wt.)
Fructose	0.5	114.1	4.6
Glucose	0.9	114.1	4.6
Raffinose	2.0	—	—
Sucrose	41.8	24.8	1.0
Total	45.2	253.0	10.2

Analyses of the sugars found in the pollen and in the pollen tissue culture are given in Table V. Sucrose predominated in the pollen and fructose and glucose in the tissue. Raffinose was found only in the pollen. The differences in total sugars between the tissue culture and the pollen were attributed to the large amount of wall material in the dry pollen and to the hydrated nature of the tissue culture.

The growth response of the pollen tissue to media containing various sugars was tested. Seven sugars were added separately to the standard growth medium to compare their utilization with the sucrose normally used. The results are shown in Figure 1. Sucrose and raffinose were the best energy sources, followed by lactose, glucose, and galactose. Fructose was very poor; xylose and arabinose were not utilized at all by the tissue. The poor growth on fructose, and possibly some of the other sugars, may be due to degradation during autoclaving.

About six times as much RNA-P and more than twice as much DNA-P was found in the pollen as in the tissue culture (Table VI). These differences must be interpreted by considering the fact that large amounts of

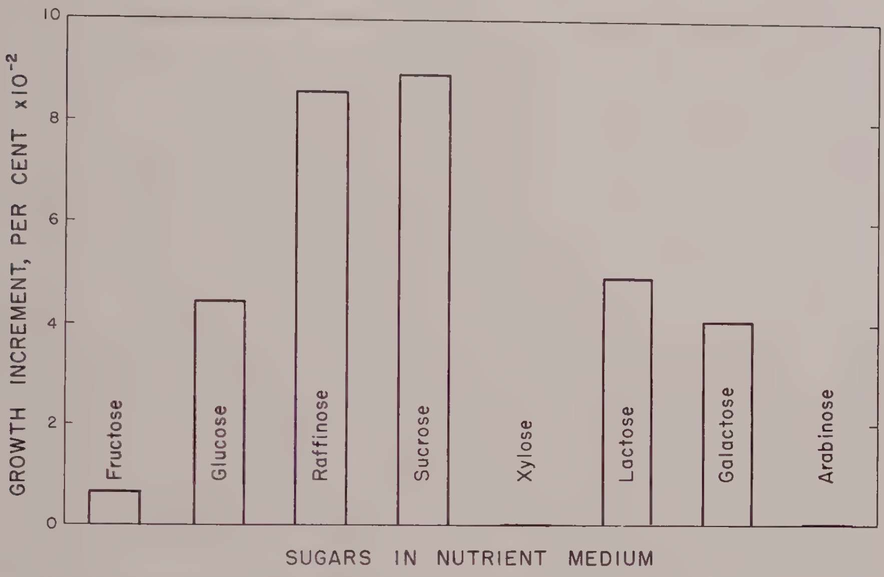

FIGURE 1. The growth of *Ginkgo* pollen tissue on media containing various sugars. The medium contains the amino acid arginine, inorganic salts, vitamins, and naphthaleneacetic acid (22); the sucrose normally included in the medium was replaced by the other sugars at a concentration of 2 per cent. Sugars were added before autoclaving.

inert pollen wall material were found in the alcohol-insoluble residue from the pollen, but that not nearly as much starch and cell wall materials were found in the alcohol-insoluble residue from the tissue. Nevertheless, the RNA-P/DNA-P ratios indicated that the pollen contained more RNA-P and the tissue culture more DNA-P.

An unknown neutral substance which makes up about 1 per cent of the dry weight of pollen was isolated from the ethanolic extract after passage through both Dowex 50W-X8, hydrogen form, and Dowex 1-X10, acetate form, resins. The unknown was composed of small, yellow, needle-shaped crystals, m.p. 188° C. and contained no phosphorus, nitrogen, or halogens. It was soluble in hot water or ethanol and exhibited pearly opalescence in water. The compound was stable to heat (45 min. at 105° C.) and was

TABLE VI

RNA-P AND DNA-P CONTENT OF GINKGO POLLEN AND POLLEN TISSUE CULTURE

	μG. RNA-P/100 mg. alc.-insol. residue	μG. DNA-P/100 mg. alc.-insol. residue	RNA-P/DNA-P
Pollen	445.0	59.7	7.4
Tissue culture	68.8	27.0	2.5

insoluble in common organic solvents such as benzene, chloroform, carbon tetrachloride, acetone, camphor, and ethyl ether. Carbon-hydrogen determination gave C = 51.20–52.20 per cent; H = 5.07–5.17 per cent. There was a shift in ultraviolet absorption with the addition of alkali. Upon chromatography, the Rf was 0.50 in isoamyl alcohol—pyridine—water (3:2:1.5 v/v). The available data suggested a glycoside or a naphthoquinone. However, co-chromatography of juglone, 2-methyl naphthoquinone, and 2-methoxy-1,4-naphthoquinone (12) gave negative results.

DISCUSSION

Similarities and differences in the biochemical composition of *Ginkgo* pollen, germinated pollen, and the tissue culture were found. Citric and malic acids were high in both the pollen and the tissue. The metabolically inactive pollen was found to store sucrose as the main sugar, whereas the tissue contained principally fructose and glucose. This difference may be attributed to the fact that the invertase in the dry pollen was inactive, hence the stored sucrose was not hydrolyzed. Most pollens (5, 10), including *Ginkgo* (19), are known to have invertase and some evidence for this enzyme in the tissue culture has been described.[2]

Although the age of a tissue culture (or any tissue) will affect the content of free amino acids,[2] the amino acid composition of the pollen tissue culture was found to resemble germinated pollen more closely than it did the ungerminated pollen, the nucellus, or the leaves.

Differences in the amounts and kinds of protein amino acids of the tissue culture and germinated pollen were large. Cysteic acid, phenylalanine, and arginine were absent from germinated pollen and present in the tissue. The amounts of other amino acids were quite variable. Such variations were not found between fractions of seed proteins of *Ginkgo* by Ono (15). Globulin, glutelin, water-soluble globulin, and albumin proteins showed rather consistent high levels of glutamic acid, arginine, and aspartic acid and lesser amounts of leucine and alanine.

Qualitative assays of free and protein amino acids of *Ginkgo* seeds and germinating embryos were reported by Hatano (6). Arginine was a major constituent of the free amino acids, and asparagine was absent from the seeds but present in the seedlings. Hatano (7, 8) later reported the qualitative changes in the free amino acids of leaves and endosperm during the growing season. Arginine was absent from the leaves but present in the endosperm. Asparagine was low in the leaves and seeds, but aspartic acid was abundant in both tissues. The present quantitative results for *Ginkgo* tissues indicate a high asparagine content for pollen and agreement with Hatano's results for aspartic acid in the leaves. Aspartic acid was abundant in the nucellus but low in the tissue culture and germinated pollen.

An important paper relating amino acids to pollen germination and growth is that of Sawada (18). His results indicate that, in general, some of the amino acids naturally occurring in the pollen, ovary, or stigma will stimulate pollen growth. These results were confirmed by Tupý (24) who showed that the amount of pollen tube growth in *Nicotiana alata* was directly related to the content of alanine and γ-aminobutyric acid in the ovary. Compatible crosses showed a positive correlation between amino acid content and pollen tube growth, while incompatible crosses did not.

The literature on the chemical composition of pollen has been reviewed by Lundén (13) and the physiology of pollen by Johri and Vasil (11). Most of the analyses of pollen were related to the use of pollen as bee food; mostly wind-pollinated species were analyzed. Virtanen and Kari (25) reported on qualitative assays of free amino acids in pollen and contrasted these with the amino acids in green plant parts. Substantial amounts of free proline, hydroxyproline, and pipecolic acid were found in pollen but not in the green parts of the plants; the same trend was noted for basic amino acids and amides. For *Ginkgo* pollen, the same general pattern was found. Free proline, basic amino acids, and amides were abundant in the pollen and tissue, but not in the leaves or nucellus. However, free hydroxyproline and pipecolic acid were not found. Similar results for *Pinus canariensis* pollen were reported by Havivi and Leibowitz (9).

Analytical studies by Bové, Bové, and Raveux (3) showed that tissue cultures of *Citrus limonum* contained more proline, γ-aminobutyric acid, and citric acid than did the seedlings of the plant. Citric acid was also more abundant in *Ginkgo* and *Agave* (26) tissue cultures than in the plant. Only γ-aminobutyric acid was present in *Ginkgo* but neither γ-aminobutyric acid nor proline was present in *Agave*. This variation between plant tissue cultures was also stressed by Roberts and Simonsen (17), who reported on comparisons of the free amino acids of normal and neoplastic tissues of plants and animals. They found that tumor tissue cultures of carrot, tobacco, cactus, and other tissues did not resemble each other as much in their free amino acids as did animal tumors.

Previous work on the chemical composition of *Ginkgo* has been reviewed by Sosa-Bourdouil (19). Many of the analyses were made on leaves, wood, or reproductive parts of male and female trees; a wide variety of substances was reported. Ginkgolic acid, ginkgol, bilobol, cerylic alcohol, two flavones (apigenol and ginnol), and unidentified sterols were among these. Shikimic and quinic acids and raffinose, reported here for the pollen, were previously known as constituents of other parts of the tree (1, 16, 19). The ribonucleic acid content was not determined before, but the deoxyribonucleic acid of *Ginkgo* pollen was known to be high compared to other gymnosperms (19).

Ball (2) has reported the utilization of sugars by *Ginkgo* embryos grown *in vitro*. Of the sugars absorbed through the cotyledons, glucose, sucrose, fructose, raffinose, and galactose, in that order, gave the best root growth.

Shoot development depended on an adequate nitrogen source, such as glutamine. These results were based on media prepared without autoclaving the sugars. The growth of the tissue culture from *Ginkgo* pollen was best on sucrose, raffinose, lactose, glucose, and galactose, in that order, but these media were autoclaved.

The culture of tissues of higher plants *in vitro* represents a gross change in many of the factors which influence growth and function. The absence of adjacent tissues, the artificial medium, and the closed nature of the culture system are all part of the new environment. It is therefore not surprising that analyses of the tissue cultures and the parent plant parts should show differences as well as similarities. Nevertheless, the tissues have been shown to retain some of their inherent capabilities, such as the production of specific compounds (27) or the ability to regenerate a whole plant (4, 20). The general conclusion already evident from the work on *Agave* (26) and confirmed to a large extent by the analyses of *Ginkgo* is that the tissue cultures should be considered as a type of growth which resembles the original tissue only to a limited extent. The differences that exist are great in some instances, but no definite pattern has emerged. The present techniques and knowledge of tissue cultures have not yet determined the degree of control which may eventually be exercised over cells grown *in vitro*. It remains to be seen whether normal or abnormal cell functions may be regulated to utilize the peculiar enzymatic, physiological, or biochemical attributes of cells grown *in vitro*.

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RESULTS OF TEN YEARS' WORK (1951–1960) ON THE EFFECT OF FLUORIDES ON GLADIOLUS

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SUMMARY

Results of the effects of fluorine (F) compounds on the growth and development of *Gladiolus* sp. are summarized for a period covering the decade 1951 to 1960.

Tip burn and accumulation of F in control and hydrofluoric acid (HF) fumigated plants varied considerably with the variety of gladiolus, cultural site, age of plant, and age of leaf. Control plants in the area where experimental fumigations with fluorides were carried out had more tip burn and accumulated more F than plants 0.5 to 1.3 miles distant. This was correlated with less atmospheric F at the distant locations. Gladiolus in the 5- to 7-leaf stage exhibited more tip burn and accumulated more F than plants in the 3-leaf stage. Middle-aged leaves generally exhibited the most tip burn, but the older leaves accumulated more F than the younger leaves.

In general, the more resistant varieties accumulated the most F and susceptible varieties the least amount, irrespective of the flower color of the variety. This applied both to nonfumigated plants and plants fumigated with HF. Tip burn and accumulation of F were greater at higher concentrations of HF. Increase in tip burn was correlated with dosage up to 2 to 4 times the threshold level. The dosage-response curve for moderately resistant Elizabeth the Queen was significantly flatter than for the susceptible Snow Princess variety. There was a highly significant regression of tip burn on F content of leaves in both varieties. There was no relation between susceptibility and number and location of stomata.

At levels of HF up to about 2 parts per billion (p.p.b.) of air, which caused 5 to 8 cm. of tip burn, there was no significant effect on dry weight of tops or corm yield in the varieties Snow Princess and Elizabeth the Queen, but the production of flower spikes was significantly less in this series of experiments. There was no close correlation between length of tip burn and size of corm produced by the same plant.

Within one week after exposure to HF, Snow Princess had lost up to 40 to 50 per cent of the F from aboveground parts of the plant, while control plants gained in F. Elizabeth the Queen lost little (av. 10 per cent) or no F. The loss was generally greater from small than from large plants. Extensive replicate sampling showed considerable variation in the loss of F from potted plants.

INTRODUCTION

Investigations of the effects on vegetation of various air pollutants, such as sulfur compounds, hydrocarbons, chlorine, and other halogens, were started in 1926. During the period of World War II preliminary investigations were made on the effect of fluorides on plants, but an intensive research program on fluorides did not start until 1951. During the past 10 years more than 150 species and varieties of plants have been fumigated

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or otherwise treated with F compounds under various experimental conditions. Since gladiolus was known to be among the more susceptible species (1, 3, 6, 13), it was studied intensively and was used as an indicator plant in many tests with other species. More than 2500 analyses have been made of the F content of gladiolus tissue.

This report summarizes the results of investigations carried out during the years 1951 to 1960 relating to the effects of F compounds on the growth and development of gladiolus.

MATERIALS AND METHODS

Plant material. Gladiolus corms purchased as size No. 1 (1½- to 2-inch diameter) were planted in field plots, in 7- or 8-inch clay pots, and in 8-inch metal pots. If the diameter of corms varied considerably, they were sorted into different size groups at the time of planting so that each pot or plot contained the same number of a given size.

In most tests four corms per pot were planted at a depth of about 4 inches. Before treatment the pots were sorted into groups according to the number and relative size of shoots which had emerged. Each group of pots was randomized separately for the various treatments. A randomized scheme was also used for collecting samples of aboveground parts of gladiolus planted in plots.

In some of the experiments potted gladiolus were fumigated in plots with other species. The loss of F from gladiolus was compared with the loss from milo maize, varieties Martin's and Hegari (*Sorghum* sp.); sweet corn, variety Marcross (*Zea mays* L.), in field plots; and from potted tomatoes, variety Bonny Best (*Lycopersicon esculentum* Mill.), exposed to the same levels of HF.

Fumigation equipment. Portable houses covered with Vinylite plastic or polyethylene film of 0.004-inch thickness (Bakelite Co., Division of Union Carbide Corp., Bound Brook, New Jersey) were used for fumigating plants in the field. The use of polyethylene covered houses (Fig. 1) started in 1960. The plastic covering was held in place by means of flexible cables fitted into the slotted arched aluminum ribs that constituted the roof and side wall framework of the house (Lord & Burnham, Division of Burnham Corp., Irvington-on-Hudson, New York). The volume of each house was approximately 500 cubic feet and covered an area 8 feet square. Plants inside the house were exposed to a steady flow of air scrubbed by atomized water and supplemented by injected HF or fluosilicic acid (H_2SiF_6). In 1957 and thereafter HF was used exclusively. The injection equipment and procedures for fumigating plants from 1954 to 1960 have been described in previous reports (7, 9, 15).

In 1960 the injection equipment described by Hill *et al.* (5) was used in exploratory tests for introducing fluorides into one of the portable houses.

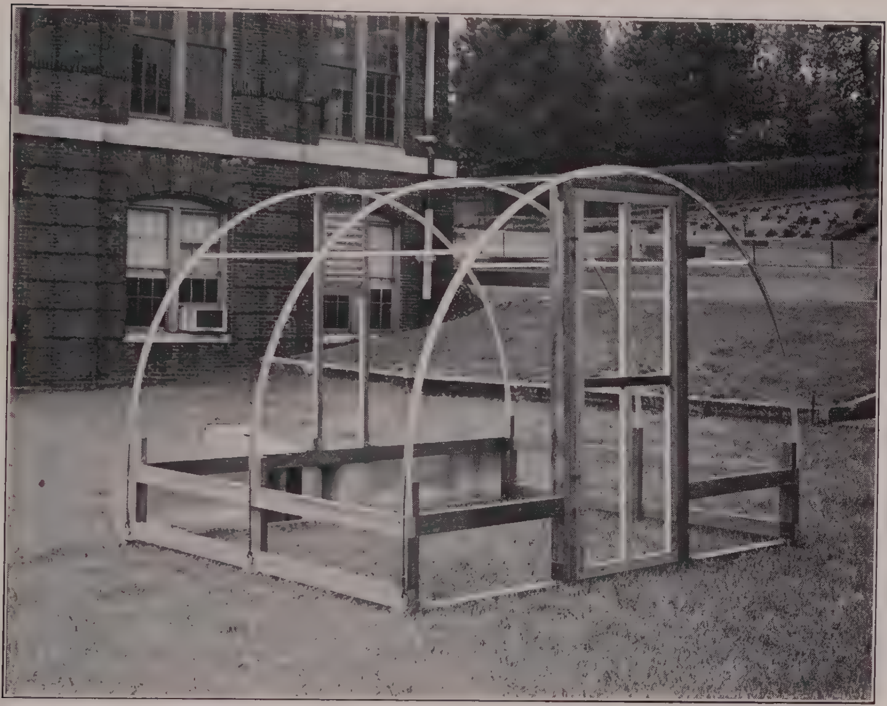

FIGURE 1. Frame of quonset type fumigation chamber used in 1960. Plastic film covering is removed to show the following structural features: basal aluminum plenum and connecting duct for handling and distributing incoming air mixture shown in rear and exit grill above, arched slotted aluminum ribs with vinyl covered flexible steel cables used to secure plastic covering, and entrance door in front.

Certain modifications were made in order to adapt the equipment for use with the large diameter air delivery duct used in all portable houses. In view of its compactness, ease of operation, and reproducible results, it is contemplated using only this injection equipment in the future. The modified equipment will be described in a paper to appear in a later issue of the contributions.[3]

Determination of fluorides in plant tissue and air. Plants were harvested and records taken of the fresh and dry weights of leaves and other aboveground parts. Plant tissue was cut into small pieces, dried at 85° C., ground in a standard No. 3 Wiley mill (Arthur H. Thomas, Philadelphia, Pennsylvania), blended, and aliquots of 3 to 5 g. were digested for F analysis. There was no loss of F from dried leaf tissue (4) which is in agreement with the results of Thomas and Hendricks (13). The procedures used for the determination of fluorides in plant tissue and in air were based on the Willard and Winter method (14). A detailed description of the analytical methods used in this laboratory for the determination of fluorides is given in previous reports (4, 8, 10, 11). These and procedures currently employed will be summarized in a subsequent paper.[3]

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[3] Mavrodineanu, R., J. Gwirtsman, D. C. McCune, and Clark A. Porter. Summary of procedures used in the controlled fumigation of plants with volatile fluorides and in the determination of fluorides in air, water, and plant tissues. (In manuscript.)

Significant differences.[4] Comparisons of the significance between means in various tabular data are based on the analyses of variance and the Tukey test (12, p. 251). The required significant difference at the 5 per cent level for the Tukey test is appended to the pertinent tables. It should be noted that the commonly used least significant difference is applicable only for the comparison of two treatments. While the Hartley sequential test (12, p. 251) is somewhat more powerful than the Tukey test and may detect the greater number of differences, it has not been used because of its complexity in tabular presentation.

RESULTS AND DISCUSSION

EFFECT OF FLUORIDES ON GROWTH AND YIELD

Air sampling. Samples of air were obtained by passing a measured volume of air through deionized distilled water (8). The volume of air collected for analysis depended upon the expected concentration of HF. A rate of about 12 cubic feet per hour was used for collecting a 24-hour sample containing 1 to 10 p.p.b. v/v HF. The average results of four or five 24-hour collections were used as a basis for expressing the average concentration of HF in most fumigation tests. Air in one or two portable houses used for HF injection was sampled continuously for comparison with the average results of 24-hour sampling. Results for house No. 4 are shown in Table I. The washed air in control portable houses was sampled less frequently because earlier results had shown <0.05 p.p.b. HF (Table I). Rates up to 40 cubic feet per hour were used for shorter periods of sampling. Results of collections made during the day (11 A.M. to 2 P.M.) and at night (12:01 to 4 A.M.) showed that the concentration of HF was consistently higher during the day (av. 1.5 times) than at night in the same houses when the average night temperatures were 14° to 19° F. lower than day temperatures during the sampling periods (Table II). Diurnal differences in fluoride concentration can be accounted for by the greater adsorption of HF on the cooler surfaces at night than occurs on the warmer surfaces during the day. Results discussed later in connection with the F content of ambient air in different locations also show that the lowest concentrations of F are correlated with low temperatures. The air was sampled in fumigation chambers at or slightly above the tops of test plants. When the concentration of HF in the delivery duct was 1 to 3 p.p.b., the

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[4] All statistical analyses were carried out by Dr. S. E. A. McCallan.

TABLE I

COMPARISON OF THE AVERAGE CONCENTRATION OF HF IN ATMOSPHERE OF PORTABLE GREENHOUSES BASED ON INTERMITTENT VS. CONTINUOUS SAMPLING OF AIR (1958)*

House No. or location	Av. concn. of HF, p.p.b. v/v, in air							
	First fumigation				Second fumigation			
	Series A, upper plots							
1	0.91	0.67	0.62	0.82	0.43	0.52	0.88	0.67
2	2.69	2.07	2.55	2.84	2.11	1.84	1.92	2.03
	Series B, lower plots							
4	0.69	0.30	0.46	0.67	0.71	0.48	0.80	0.81
4**	0.60	0.78	0.40	0.71	0.62	0.46	0.63	0.75
6	3.15	3.47	2.67	2.73	2.40	2.23	2.35	2.70
Control washed air**	—	—	<0.05	<0.05	<0.05	—	<0.05	<0.05
Ambient outside air†	0.45	—	0.43	0.65	0.76	0.80	1.00	0.49

* Intermittent air analyses were obtained by direct titration of the fluorides in 24-hour samples.

** After continuous sampling for periods of 6 to 8 days, the fluorides in air samples were steam distilled.

† Absorber-distilling unit with automatic water renewal located between two series of plots was used to collect the F from 3,000 or more cubic feet of air.

TABLE II

AVERAGE CONCENTRATION OF HF MAINTAINED IN PORTABLE GREENHOUSES DURING THE DAY (11 A.M. TO 2 P.M.) AND NIGHT (12:01 TO 4 A.M.) IN FOUR SUCCESSIVE EXPERIMENTS BETWEEN MAY 17 AND JUNE 27, 1957

Approximate concn. desired, p.p.b.	Sample taken during	Mean concn. of HF, p.p.b.*					Ratio of day concn. to night**
		May 17–27	May 29–June 10	June 12–19	June 20–27	Av.	
1.00	Day	1.05	0.93	1.47	1.47	1.23	1.62
	Night	0.95	0.67	0.73	0.67	0.76	
2.00	Day	2.65	2.07	3.63	2.97	2.83	1.43
	Night	1.80	1.73	2.33	2.07	1.98	
4.00	Day	5.05	5.67	3.87	4.60	4.80	1.42
	Night	3.78	3.20	3.03	3.47	3.37	
8.00	Day	12.00	6.33	9.40	9.07	9.20	1.50
	Night	8.93	5.15	5.23	5.13	6.11	

* Mean of 3 days and mean of 3 following nights.

** Average night temperature 14° to 19° F. lower than day temperature during sampling periods.

concentration at the tops of test plants was 60 to 75 per cent lower.

Absorption distillation units (4) were used for sampling air containing low amounts of F. Results of two series of tests carried out in a fumigation chamber showed a coefficient of variation of 4.3 to 5.6 per cent (Table III). Collections of 3,000 or more cubic feet of ambient air were considered essential because of the relatively low levels of HF in the air over the experimental plots (Table IV).

The total F in air samples is expressed as p.p.b. HF. The lower values obtained with the use of a Millipore filter (Table V) indicate that particulate F compounds are present in the ambient air over the experimental plots.

Symptoms of fluoride injury. Tip necrosis was observed on all gladiolus grown in the field or in the greenhouse at Yonkers and on gladiolus in various parts of the country far removed from industries. Under natural conditions tip burn usually appeared when the plant had two to three well-developed leaves and progressed slowly and rather evenly downward with increasing age of the plant. The time of appearance and rate of increase varied with individual plants, the variety of gladiolus, and cultural conditions. Typical results for Snow Princess and Elizabeth the Queen are shown in Table VI. At the time of flowering, tip burn had extended downward one or more centimeters on some leaves.

TABLE III

RELIABILITY OF CONICAL ABSORPTION DISTILLATION UNITS IN SAMPLING AIR CONTAINING LOW AMOUNTS OF F

Replicate absorbers in chamber	Cu. ft. of air sampled*		Concn. of HF detected, p.p.b. v/v**	
	First test	Second test	First test	Second test
No. 1	3,042	3,138	0.34	0.28
No. 2	3,224	3,138	0.33	0.26
No. 3	3,311	3,138	0.31	0.28
No. 4	3,522	3,138	0.34	0.25
Mean			0.33	0.27
Coefficient of variation			4.3%	5.6%

* The rate of air flow was approximately 20 cubic feet per hour.

** The fluorides were steam distilled directly from the absorption vessel before titration.

The atmosphere in the experimental areas naturally contained trace amounts of fluorides, usually less than 0.5 p.p.b., and sometimes less than 0.10 p.p.b., depending on the temperature. These traces of atmospheric fluorine are believed to be the principal cause of the tip burn just described. Under natural conditions tip burn is indistinguishable from that induced by fumigation with relatively low levels of HF or H_2SiF_6. Higher levels of volatile fluoride (1 to 3 p.p.b. or more) caused a more uneven necrosis

TABLE IV

F CONTENT OF AMBIENT AIR BASED ON RESULTS WITH THE CONTINUOUS ABSORBER

Sampling period, 1957	Location	Volume of air, cu. ft.	Total µg. F	Total blank µg. F	Net µg. F	P.p.b. HF by volume
Aug. 19–22	B.T.I.*	5,400	51.5	1.5	50	0.40
Aug. 23–26	B.T.I.*	5,150	28	2.0	26	0.23
Aug. 27–30	B.T.I.*	4,470	56	2.4	53.6	0.35
Sept. 9–12	B.T.I.*	5,520	55	3	52	0.43
Sept. 12–16	B.T.I.*	7,870	77	2	75	0.43
Sept. 16–19	B.T.I.*	3,500	36	3	33	0.43
Oct. 8–10	B.T.I.*	4,860	62	5	57	0.50
Oct. 10–12	Arboretum**	2,700	15.7	2.1	13.6	0.23
Oct. 12–17	Arboretum**	3,970	34	2.0	32	0.37
Oct. 17–20	Arboretum**	3,650	28.5	4.0	24.5	0.31
Nov. 1–6	B.T.I.*, only day sampling	1,830	12.5	3	9.5	0.23
Nov. 7–14	B.T.I.*	8,240	30	2.2	27.8	0.16
Nov. 14–19	B.T.I.*	6,970	29.6	2.5	27.1	0.19
Nov. 22–25	B.T.I.*	5,070	19	2.2	16.8	0.15
Nov. 25–27	B.T.I.*	3,360	6	2.5	3.5	0.05†
Oct. 25–27	Brooklyn, N. Y.	1,580	12	2.0	10	0.29

* Atmosphere over experimental plots near Boyce Thompson Institute laboratory.
** 1.3 Miles east of B.T.I.
† Temperature ≦20° F.

which extended downward along leaf margins, as shown for Snow Princess in Figure 2, or occurred as scattered areas below the necrotic tip. It is of interest that Thomas and Hendricks (13) refer to a low concentration range of 0.1 to 14 p.p.b. as that which is usually encountered in the field.

The color of necrotic leaf tips ranged from ivory or light tan through

TABLE V

RESULTS OF SAMPLING AMBIENT AIR FOR F CONTENT WITH AND WITHOUT A MILLIPORE FILTER

Date of collection, 1960	Volume of air, cu. ft.	Filter	µG. F	HF, p.p.b.	Dates of HF fumigations in area
Sept. 20–27	3,676	None	25.4	0.32	Oct. 20–27
	3,639	Present	9.7	0.12	
Sept. 27–Oct. 5	4,357	None	22.7	0.24	Oct. 4–11
	4,324	Present	15.0	0.16	
Oct. 5–13	4,140	None	18.2	0.20	
	3,867	Present	5.8	0.07	
Oct. 13–21	4,578	None	12.5	0.13	None
	4,106	Present	9.1	0.10	

various shades of brown. In general, light flowered varieties, like Snow Princess and Spotlight, exhibited lighter shades of tan or brown than red or dark flowered varieties, such as Stoplight and Valeria. Such varietal differences in the color of leaf necrosis were more pronounced on HF-fumigated plants than on control plants. The tip burn on control plants tended to become progressively darker, especially on plants which did not flower until after August 15.

Control plants having leaf tip burn seldom showed bract burn on unopened tightly compressed florets. In experimental fumigations similar bracts exposed to HF frequently developed tip and marginal burn before any burn resulting from the fumigation appeared on leaves. These results indicate that flower bracts are as susceptible as, or possibly more susceptible to HF fumigation than leaves. Additional evidence of the sensitivity of flower bract tissue was furnished by observing the effects of abnormal water stress on flower spikes during the period of most rapid elongation. Upon recovery from wilting, some of the flower spikes failed to regain a normal vertical position. Tip and marginal burn that developed on the unopened floret bracts of these spikes was indistinguishable from HF-induced flower bract burn.

TABLE VI

EXTENT OF TIP BURN ON LEAVES OF TWO VARIETIES OF GLADIOLUS IN AMBIENT AIR 29 TO 32 DAYS AFTER PLANTING*

Snow Princess			Elizabeth the Queen		
No. per pot			No. per pot		
Shoots	Leaves	Leaves with tip burn**	Shoots	Leaves	Leaves with tip burn**
5	13	4	5	13	1
6	12	5	4	10	0
5	13	4	4	11	0
7	14	9	4	11	0
5	14	4	5	11	1
5	13	11	3	7	1
7	14	5	4	10	0
5	13	6	5	14	1
Av. 5.6	13.3	6.0	4.3	12.2	0.5

* Tip burn first observed after 29 days on Snow Princess and after 32 days on Elizabeth the Queen.

** Length of tip burn <1 to 3 mm.

Flowers on control plants and on plants fumigated with relatively low levels of HF (1 to 2 p.p.b. or less) exhibited no marginal discoloration such as was observed on plants fumigated with more than 4 p.p.b. HF. Marginal bands 1 to 3 mm. in width were whitish on the lavender petals of Elizabeth the Queen, water soaked on white petals of Snow Princess, and yellowish-orange on the red petals of Valeria. Open flowers were more affected than flower buds showing color.

Evaluation of tip burn. Tip burn was evaluated in several ways. In most cases the length of burn on each leaf was measured from the tip and expressed as the average or the combined length of tip burn per plant per treatment. Measurements in some tests were based on the average length of burn on two or three leaves. If the necrotic area was a solid triangle, the altitude represented the length of tip burn; otherwise, an estimate was made of the altitude of a triangle equal in area to that of the necrotic region. The length of tip burn was measured in centimeters as a matter of convenience. The relation of length and corresponding area of tip burn to length and area of different aged Snow Princess leaves on flowering plants is shown in Table VII for plants grown in 8-inch metal pots without addition of fertilizer to the soil.

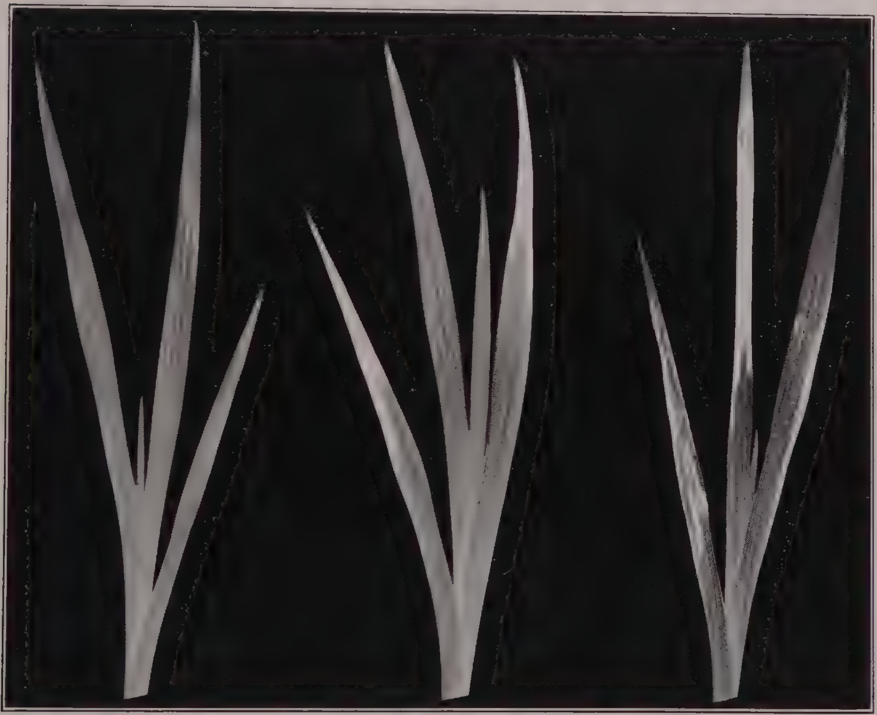

FIGURE 2. Comparative symptoms of fluoride tip burn and SO_2 necrosis on gladiolus var. Snow Princess. Leaves in the center were exposed to 8 p.p.b. HF for 6 days and contained 75 p.p.m. of F at harvest. Those on the right were exposed to 1.5 p.p.m. of SO_2 for 7 hours on each of 3 days. Control on the left.

Tip burn and accumulation of F under natural conditions. The length

TABLE VII

RELATION OF LENGTH AND AREA OF TIP BURN ON LEAVES OF DIFFERENT AGES TO TOTAL LENGTH AND AREA OF LEAVES ON SNOW PRINCESS GLADIOLUS DURING FLOWERING

Length of tip burn, cm.	Area, cm.^{2}, of tip burn on leaves of different ages*							Tip burn as % of total leaf area
	1	2	3	4	5	6	Av. per leaf	
1	3	2	1	2	2	2	2	0.4
2	7	5	5	5	3	5	5	1.0
4	24	17	16	16	8	13	16	3.2
6	41	33	29	28	23	25	30	6.0
8	68	53	47	46	39	38	49	9.8
10	94	79	72	67	58	56	71	14.2
15	154	158	135	128	114	96	131	26.2
	Length and area of leaves of different ages**							
Mean length, cm.	36	46	48	48	51	46	46	—
Mean area, cm.^{2}	413	587	632	548	503	316	500	—

* Listed in order from oldest (1) to youngest (6).
** Averages of five replicates.

of tip burn on gladiolus leaves and the F content of 10-cm. leaf tips varied according to the variety, age, and site of culture of the plants, and age of the leaves. Results for five varieties in Table VIII show that the combined length of tip burn on all leaves per plant increased with the age of the plants from little or none at the 3-leaf stage in July, to noticeable amounts at the 5- and 7-leaf stages in August. Elizabeth the Queen developed the least amount and Snow Princess and Valeria the most. Varieties Spic and Span and Spotlight exhibited intermediate amounts of tip burn. Plants near the laboratory developed more tip burn than those grown on the farm (0.5 mile east) or at the arboretum (1.3 miles east). Measurements of tip burn on the first three leaves showed the same relative differences between varieties at the three locations.

The relation between the age of leaf and length of tip burn is shown for variety Snow Princess in Table IX. On control plants with five to six well-developed leaves most tip burn occurred on the middle-aged leaves and least on the oldest and youngest leaves. Individual plants showed considerable variation in the length of tip burn on leaves of different age (control No. 1), but the average values were highest and nearly the same for the third and fourth leaves on the ten plants measured in each of the three control plots. The average tip burn for all leaves per plant ranged from 0.6 to 3.6 cm., but the average for all leaves on ten plants was nearly the same for each of the three control plots, namely 1.9, 2.5, and 2.3 cm. The length of tip burn on the fourth leaf was considered by Compton and Remmert (2) as indicative of differences due to location of gladiolus outward from an aluminum reduction plant. The results in Table IX indicate

TABLE VIII

EXTENT OF TIP BURN ON FIVE VARIETIES OF GLADIOLUS HELD IN AMBIENT AIR AT DIFFERENT LOCATIONS IN YONKERS

Variety, planted May 28, 1956	Leaf stage and date measured	Length of tip burn, cm., on *all leaves**					
		North of lab.	Terrace west of lab.	Pots in terrace soil	Farm 0.5 mi. east	Arboretum 1.3 mi. east	Mean
Elizabeth the Queen	3 (July 11)	0	0	0	0	0	0.0
	5 (Aug. 1)	2	8	1	2	1	2.8
	7 (Aug. 29)	11	17	9	8	6	10.2
	Mean	4.3	8.3	3.3	3.3	2.3	4.3
Spic and Span	3 (July 11)	0	1	0	0	0	0.2
	5 (Aug. 1)	5	6	4	2	0	3.4
	7 (Aug. 29)	10	16	13	8	9	11.2
	Mean	5.0	7.8	5.7	3.3	3.0	4.9
Spotlight	3 (July 25)	1	2	1	1	1	1.2
	5 (Aug. 10)	6	6	4	3	2	4.2
	7 (Aug. 31)	12	13	10	8	6	9.8
	Mean	6.3	7.0	5.0	4.0	3.0	5.1
Snow Princess	3 (July 11)	1	1	2	1	0	1.0
	5 (Aug. 1)	11	8	8	4	1	6.4
	7 (Aug. 22)	16	15	15	8	5	11.8
	Mean	9.3	8.0	8.3	4.3	2.0	6.4
Valeria	3 (July 11)	1	2	3	0	1	1.4
	5 (Aug. 1)	10	6	9	4	3	6.4
	7 (Aug. 22)	20	16	17	8	8	13.8
	Mean	10.3	8.0	9.7	4.0	4.0	7.2
Mean 5 varieties		7.1	7.8	6.4	3.8	2.9	5.6

* Same ten plants measured at three different stages of development.

that reproducible results are most likely attained by measurement of length of tip burn on middle-aged leaves or on all leaves of several plants. Results of measuring tip burn on thousands of gladiolus plants over a period of ten years have shown that the average length of tip burn on two or three middle-aged leaves gave about the same relative differences between varieties and treatments as the average values for all leaves per plant. However, the latter measurements gave a better estimate of the relative amount of necrotic tissue per plant, even though it is a more time-consuming procedure.

There was less F in the tips of leaves (10 cm. in length) collected from plants in the 3-leaf stage than for older plants in the 5- to 7-leaf stages (Table X). The level of F was highest in leaf tips of Elizabeth the Queen

TABLE IX

EXTENT OF TIP BURN ON DIFFERENT LEAVES OF SNOW PRINCESS GLADIOLUS BEFORE AND AFTER EXPOSURE TO HF FOR 6 DAYS

	Length of tip burn, cm., according to age of leaf (1=oldest)													
	Before treatment							Four days after treatment						
	1	2	3	4	5	6	Av.	1	2	3	4	5	6	Av.
	Control No. 1							Exposed to scrubbed air						
	1	9	3	4	0	—	3.4	2	10	3	5	1	1	3.7
	0	0	3	3	0	—	1.2	1	2	6	3	0	0	2.0
	3	3	3	4	0	—	2.6	3	4	3	4	2	1	2.8
	2	1	2	4	0	0	1.5	3	4	2	4	1	2	2.7
	0	2	2	5	2	1	2.0	3	2	7	5	2	2	3.5
	0	0	5	1	1	0	1.2	2	2	5	1	1	1	2.0
	0	0	2	1	0	—	0.6	0	0	2	2	1	1	1.0
	0	2	3	3	4	0	2.0	0	2	3	3	4	1	2.2
	0	6	6	5	1	—	3.6	0	7	7	8	1	1	4.0
	1	0	2	1	1	1	1.0	1	1	4	5	3	3	2.8
Av.	0.7	2.3	**3.1**	**3.1**	0.9	0.2	1.9	1.5	3.4	**4.2**	**4.0**	1.6	1.3	2.7
	Control No. 2							Exposed to 1 p.p.b. HF						
Av.	1.0	2.5	**3.2**	**4.1**	3.0	0.9	2.5	2.4	5.1	**6.4**	**6.9**	5.8	3.9	5.1
	Control No. 3							Exposed to 2.8 p.p.b. HF						
Av.	1.4	1.9	**3.7**	**3.5**	1.8	0.8	2.3	8.1	12.4	**19.2**	**18.2**	14.2	5.6	13.0

and Spotlight and lowest in the leaf tips of Snow Princess. Thus, Elizabeth the Queen, though less susceptible to tip burn, accumulated more F than Snow Princess. The accumulation of F by these five varieties in different cultural sites (Table X) followed the same trend as tip burn on the same plants (Table VIII), namely lesser amounts for plants at the farm and arboretum as compared with those near the laboratory.

Leaf tips of different age (three oldest and four youngest) had less F in plants grown at the farm and arboretum than in plants grown near the laboratory. Results of these tests also showed that the three oldest leaf tips accumulated more F than the four youngest leaf tips and that the level of F in tips of Elizabeth the Queen leaves was several-fold higher than in leaf tips of Snow Princess.

The lesser amount of tip burn and lower levels of F in gladiolus grown at the farm and arboretum are attributed to a lower concentration of fluorides in the air. Analyses of the atmosphere for total fluorides during the period October 8 to 12, 1957, revealed more F near the laboratory (0.50 p.p.b.) than at the arboretum (0.23 p.p.b.). The lowest concentrations of F calculated as HF measured at above freezing temperatures in ambient air near the laboratory were 0.15 to 0.19 p.p.b. in late November, 1957,

TABLE X

ACCUMULATION OF F IN THE TIP 10 CM. OF LEAVES COLLECTED AT THREE DIFFERENT STAGES OF DEVELOPMENT FROM FIVE VARIETIES OF GLADIOLUS GROWING IN AMBIENT AIR AT DIFFERENT LOCATIONS (SEE ALSO TABLE VIII)

Variety	Leaf stage and date sampled	F, p.p.m., according to variety, age (leaf stage), and location of plants					
		North of lab.	Terrace west of lab.	Pots in terrace soil	Farm 0.5 mi. east	Arboretum 1.3 mi. east	Mean
Elizabeth the Queen	3 (July 11)	43	32	26	17	7	25
	5 (Aug. 1)	57	27	28	40	13	33
	7 (Aug. 29)	52	31	38	38	13	24
	Mean	51	30	31	32	11	31
Spic and Span	3 (July 11)	26	13	14	8	7	14
	5 (Aug. 1)	28	23	28	11	6	19
	7 (Aug. 29)	25	11	15	19	6	15
	Mean	26	16	19	13	6	16
Spotlight	3 (July 25)	50	30	40	24	12	31
	5 (Aug. 10)	49	30	45	15	16	31
	7 (Aug. 31)	65	38	36	53	11	41
	Mean	55	33	40	31	13	34
Snow Princess	3 (July 11)	21	12	11	6	2	10
	5 (Aug. 1)	25	24	21	13	5	18
	7 (Aug. 22)	22	22	20	11	4	16
	Mean	23	19	17	10	4	15
Valeria	3 (July 11)	40	21	29	17	7	23
	5 (Aug. 1)	43	38	32	21	16	30
	7 (Aug. 22)	33	32	23	20	10	24
	Mean	39	30	28	19	11	25

about a month after HF fumigations in the field had ceased (Table IV), and 0.10 to 0.26 p.p.b. during the period April 4 to June 13, 1960, before HF fumigations in the field started. Although the intervals between fumigations near the laboratory were seldom longer than one week, in one instance there was an interval of four weeks (August 8 to September 6), during which time four successive collections of ambient air showed, respectively, 0.42, 0.30, 0.30, and 0.57 p.p.b. HF. When field plots in the same area were fumigated with HF (July 12 to 19, 19 to 26, August 1 to 8, and September 8 to 15), the concentrations of F in ambient air were, respectively, 0.40, 0.65, 0.41, and 0.49 p.p.b. HF. These results show no consistent effect of the HF fumigations on the F content of ambient air near the laboratory.

Likewise, there was no effect of HF fumigations on the F content of ambient air at the farm where the concentrations of atmospheric F were considerably lower. For example, during HF fumigations at the farm (August 8 to September 6), the concentrations of atmospheric F were 0.06 to 0.15 p.p.b. HF (av. 0.11 p.p.b.) and in the absence of fumigations (July 12 to September 15) 0.09 to 0.22 p.p.b. HF (av. 0.13 p.p.b.). Thus, the considerably higher concentrations of atmospheric F near the laboratory cannot be ascribed to HF fumigations in the area. There was, however, an effect of temperature on the F content of ambient air near the laboratory. Samples of ambient air collected April 24 to May 8, May 8 to 23, June 1 to 13, 13 to 27, and June 28 to July 11 showed, respectively, 0.10, 0.21, 0.29, 0.33, and 0.35 p.p.b. HF. The average monthly temperatures (° F.) were, respectively, April 45°, May 55°, June 65°, and July 78°. Thus, the lowest levels of F (0.10 and 0.21 p.p.b.) were correlated with the lowest temperatures (45° and 55°). A similar correlation between F levels and temperature was shown by the results of analyzing the F content of the atmosphere in portable greenhouses during fumigations with HF (Table II). Results of three successive 7-day collections of air in the greenhouse during May showed, respectively, 0.045, 0.053, and 0.028 p.p.b. HF.

TABLE XI

ABSORPTION OF F BY LIME-TREATED FILTER PAPERS AND SPANISH MOSS AT DIFFERENT LOCATIONS IN 1957

Dates of exposure		Amount of F, p.p.m.			
Started	Terminated	Air station B.T.I. plots	Near gas house B.T.I.	Farm	Arboretum
Filter papers protected from rain (partially enclosed)					
Feb. 15	May 1	6	7	3	12
May 1	June 1	12	13	12	6
June 1	July 1	7	5	7	6
July 1	Aug. 1	4	1	1	1
Aug. 1	Sept. 1	6	7	5	3
Sept. 1	Oct. 1	8	7	4	4
Oct. 1	Nov. 1	4	4	5	3
Nov. 1	Dec. 1	14	23	18	13
Dec. 1	Jan. 1	16	17	10	10
Av.		9	10	7	6
Spanish moss hung in the open*					
Aug. 1	Sept. 1	18	19	14	18
Aug. 1	Oct. 1	33	23	18	20
Aug. 1	Nov. 1	29	25	18	26
Aug. 1	Feb. 1	20	24	29	24
Av.		25	23	20	22

* Av. F content at start, 13 p.p.m.

The accumulation of F by lime-treated filter papers protected from rain gave less consistent results throughout the year than the continuous absorber, but the trend was the same in most periods of measurement in showing less at the farm and arborteum than near the laboratory (Table XI). One notable difference was that filter papers accumulated less F during the growing season (June 1 through October) than during November and December, which is the reverse of the results obtained with the continuous absorber. It is considered probable that the filter papers absorbed or retained less of the volatile fraction of atmospheric F during the high temperature period (June through October) than at the lower temperatures during the remainder of the year (November through May). Nevertheless, the trend during the period July 1 to October 1 was similar for the greater accumulation of F by lime-treated filter papers and more extensive tip burn on gladiolus near the laboratory as compared with results at the farm and arboretum.

TABLE XII

ABSORPTION OF FLUORIDES BY SPANISH MOSS AND LIME-TREATED FILTER PAPER FROM AIR CONTAINING DIFFERENT AMOUNTS OF F

Fumigation treatment		F content, p.p.m.	
F, p.p.b.	Duration, days	Filter paper	Spanish moss
None*	6.5	2	12
1.7	6.5	30	56
4.7	6.5	256	170
None*	7	1	14
2.1	7	78	128
8.2	7	130	281
None*	8	3	13
1.5	8	37	55
6.8	8	162	275

* Fumigated with washed ambient air.

As a general rule Spanish moss accumulated more fluorine than filter papers (Table XII). The total amount depended upon the concentration of HF in the air and the duration of exposure. The actual amounts cannot be converted into potential absorption by gladiolus since the varieties differ so much in their rate of accumulation and there is a potential source of error under field conditions that would arise from accumulation of particulate matter on the rough surface of the moss.

EFFECT OF HF ON TIP BURN AND ACCUMULATION OF F

Tip burn on plants fumigated with HF varied with the variety much the same as tip burn on nonfumigated plants, but, with increasing concentrations of HF, differences in tip burn of the several varieties were more pronounced than in control plants. In 1953 the relative susceptibility of 24 varieties of gladiolus was determined in six experiments after exposure of the plants for 6 to 7 days to an average of 1.3 to 9 p.p.b. HF. The varieties in each experiment were ranked according to the amount of tip burn from the most severely affected to the least marked. The actual amount of tip burn ranged from an average of <2.5 cm. in the more resistant varieties to 9 cm. in the more susceptible varieties when exposed to 1.3 p.p.b. HF and 3.8 to 18.5 cm. in resistant and susceptible varieties, respectively, when exposed to 9 p.p.b. The results in Table XIII show that eight varieties were classified as susceptible, eight varieties as resistant, and eight varieties as of intermediate susceptibility. In one test the 10-cm.

TABLE XIII

F CONTENT AND RELATIVE SUSCEPTIBILITY OF 24 VARIETIES OF GLADIOLUS TO TIP BURN WHEN EXPOSED FOR 6 TO 7 DAYS TO 1.3 TO 9 P.P.B. OF HF IN DIFFERENT TESTS AND RANKED ACCORDING TO RESISTANCE (1953)

Varieties used in experiments	F content, p.p.m.*	No. of times tested	No. of times ranked among			
			Most susceptible		Least susceptible	
			First 5	First 10	Last 10	Last 5
			More susceptible			
Beacon	111	6	5	6	0	0
Aladdin	110	5	4	5	0	0
Snow Princess	142	6	4	6	0	0
Leading Lady	—	4	3	4	0	0
Ethel Cave Cole	191	6	4	6	0	0
Gold Dust	180	6	2	6	0	0
Surfside	194	6	3	5	0	0
Valeria	160	6	0	4	0	0
			Intermediate susceptibility			
Eisenhower	270	6	0	1	3	1
New Europe	243	6	1	2	3	1
Zuni	185	4	0	2	2	2
Chamouny	109	6	0	1	3	1
Lady Jane	123	6	0	0	2	0
Minuet	225	6	0	0	2	0
Paul Rubens	—	4	0	0	2	2
Spic and Span	—	6	0	1	4	2
			More resistant			
Hopman's Glory	332	6	0	1	4	2
Yellow Emperor	278	4	0	0	3	2
Rosa van Lima	341	6	0	0	5	2
Stoplight	309	6	0	0	5	4
Abu Hassan	235	4	0	0	4	1
June Bells	273	6	0	0	6	1
Bloemfontein	251	4	0	0	4	3
Spotlight	310	6	0	0	6	6

* After exposure to 9 p.p.b. for 7 days.

leaf tips of plants exposed to 9 p.p.b. HF were analyzed for F. In general, the more resistant varieties accumulated the most F and susceptible varieties the least amount.

It is commonly assumed that red flowered varieties of gladiolus are more susceptible to fluorides than white flowered varieties. Tests made in 1954 with 17 varieties showed that there was no correlation between flower color and resistance to HF in gladiolus. There was a progressive increase in tip burn from <2.5 to 13 cm. after exposure to a concentration fluctuating between 4 and 21 p.p.b. of HF for 4 days. The varieties, in order of increasing length of tip burn and the color class, were: Bloemfontein (salmon), Spotlight (yellow-cream), Morning Kiss (white), New York (red), Spotlight (red), June Bells (white), Red Fox (red), Gold Dust (yellow), New Europe (red), Elizabeth the Queen (lavender), Eisenhower (red), Hopman's Glory (yellow), Snow Princess (white), Beacon (red), Valeria (red), White Gold (cream), and Holland's Glory (salmon-pink). It is to be noted that both red and white colored varieties appeared in the resistant (first five) and susceptible (last five) groups.

Effect of duration of exposure and intermittent exposure. Gladiolus, like most other species tested, had a decided dosage response to fluorides in the air. Both the accumulation of fluorides in the plant and the extent of marking were greater at higher concentrations of HF. The rate of increase of tip burn was almost directly correlated with dosage in the range of dosages of up to 2 to 4 times the threshold. Further increase in dosage had a correspondingly less obvious effect, so the relationship seemed to follow a more or less semi-logarithmic relationship.

Increase in dosage by prolongation of exposure often had an entirely different effect from an increase in concentration of fluorides. Theoretically, twice as much exposure at half the concentration should give the plant an equal or better chance to take up fluorides and respond to them. However, shock effects from high concentrations for short periods and detoxication effects of the host may alter the time-dosage response. Because of this, several time-dosage tests were made using either continuous or intermittent applications of HF to the air.

One of the factors involved in continuous exposure over long periods of time is a possible synergistic or antagonistic effect to F toxicity from other classes of compounds in the air. Although it has not been possible to try a large number of combinations of dosages, times, and toxicants, a survey was made of some representative situations.

Tests on time-concentration studies were made in triplicate at four dosages of HF on ten gladiolus varieties that ranged from susceptible to resistant. At the highest concentration (10 p.p.b.) plants of each variety were exposed simultaneously for 3, 6, or 9 days. In the shorter exposure periods, duplicate lots of plants were held for the first and last 3-day periods of the 9-day experiment or for the first and last 6-day periods so as to compensate for any changes caused by the minor but inevitable fluctuations in concentration of HF due to mechanical imperfections of the system or to changes in climatic conditions. Since the duplicate treatments made at the beginning and end of the experiment gave essentially identical results, the data from them were pooled in composing Table XIV.

The data show a very clear dosage-response reaction. When the dosage was trebled during the first 2 days (from 1.2 to 3.7 p.p.b.), the amount of tip burn was increased on the average about 27 per cent. The amount of increase was progressively larger as one proceeded from the more resistant (19 per cent for the first three varieties) to the more susceptible (30 per cent for the last three varieties). A 50-fold increase in dosage for 9 days (from 0.2 to 10 p.p.b.) increased tip burn about 370 per cent.

TABLE XIV

EXTENT OF TIP BURN ON TEN VARIETIES OF GLADIOLUS EXPOSED TO DIFFERENT CONCENTRATIONS OF HF IN TREATMENTS EXTENDING 2 TO 9 DAYS

Variety	Av. length of tip burn, cm., after treatment						
	0.2 P.p.b. for 9 days	1.2 P.p.b. for 2 days	3.7 P.p.b. for 2 days	10 P.p.b. for 3 days	10 P.p.b. for 6 days	10 P.p.b. for 9 days	Av.
Algonquin	3.0	3.0	3.0	5.5	7.0	9.0	5.1
Elizabeth the Queen	3.0	5.0	9.0	7.5	11.5	11.0	7.8
Yellow Emperor	2.5	9.0	8.0	7.5	13.0	21.0	10.2
Zuni	4.0	7.0	9.0	9.5	14.0	21.0	10.8
Minuet	2.5	7.0	8.0	11.0	13.5	23.0	10.8
Picardy	3.5	7.0	9.0	8.5	17.5	22.0	11.3
Surfside	6.0	8.0	11.0	10.5	18.0	18.0	11.9
Snow Princess	5.5	10.0	13.0	10.5	18.0	18.0	12.5
Valeria	4.0	8.0	12.0	12.5	19.0	22.0	12.9
Aladdin	6.0	15.0	18.0	13.5	20.5	22.0	15.8
Av.	4.0	7.9	10.0	9.6	15.2	18.7	

Differences required for significance at 5 per cent level for means of treatments, 2.9 cm.; for means of varieties, 4.2 cm.

Likewise, there was a significant increase in tip burn as the duration of exposure to a constant dosage of 10 p.p.b. was prolonged from 3 to 6 and then to 9 days. The increase in tip burn for the second 3-day period was 58 per cent over the first period and for the last period was 23 per cent over the second period of 3 days. By trebling the exposure, tip burn was increased about 95 per cent, but the rate had declined with prolongation beyond the first 3 days. These data were confirmed by other tests made on four varieties during the following 2 years. The data in Table XV show that the F content of the leaf tips was increased 4- to 11-fold in different varieties by exposure to the higher concentration of HF. As a general rule, the F content of the foliage was increased about 50 per cent by prolonging the exposure from 3 to 6 days. The severity of tip burn increased roughly in proportion to changes in fluoride content of the foliage, i.e. 3- to 40-fold with increased concentration of HF in the air and about 25 per cent as either of the treatments was prolonged to 6 days.

Additional data were obtained on the susceptible variety Snow Princess and the moderately resistant Elizabeth the Queen. Each variety was tested in two locations in each of two series of experiments. Plants grown outside under field conditions were exposed for 7-day periods to washed air, a relatively low concentration of about 0.75 p.p.b. of HF, and a somewhat higher concentration of about 2.8 p.p.b. of HF. The chambers were removed for 2 weeks and then returned in the third week for another 7-day exposure, as indicated in Table XVI. Plants were removed from each plot according to a prearranged sampling schedule 7 days after the first exposure and 7 days after the second exposure. Follow-up analyses and measurements were made on two dates subsequent to the preceding, usually at intervals of 5 to 7 days each. Data on the F content of the tip 10 cm. of the leaves and of the extent of tip burn are given for Tests 1 and 2 in Table XVI.

TABLE XV

EFFECT OF DURATION OF EXPOSURE TO HF ON THE ACCUMULATION OF FLUORIDES IN GLADIOLUS LEAVES AND ON THE SEVERITY OF TIP BURN (1954)

Variety	Concn. of HF supplied, p.p.b.	F content, p.p.m., of tissue after exposure			Amount of tip burn, cm., after exposure		
		First 3 days	Second 3 days	All 6 days	First 3 days	Second 3 days	All 6 days
King Lear	Control	—	—	24	—	—	1.5
	3–16	106	94	141	9.9	3.6	4.1
	12–21	276	189	268	20.0	10.0	24.0
Commander Koehl	Control	—	—	15	—	—	5.1
	3–16	14	42	41	9.9	8.6	3.3
	12–21	82	58	122	20.0	15.0	21.0
Gold Dust	Control	—	—	16	—	—	1.0
	3–16	60	16	66	3.6	14.0	11.0
	12–21	89	74	91	13.0	10.0	16.0
Aladdin	Control	—	—	11	—	—	2.8
	3–16	23	20	32	21.0	14.0	28.0
	12–21	37	26	25	30.0	27.0	37.0
Difference required for significance at 5% level	3–16	115			Not significant		
	12–21	158			16.0		

Analysis of variance of data in Table XVI revealed that variety Snow Princess behaved entirely differently from Elizabeth the Queen both as to tip burn and the capacity to accumulate F. Because of this, it is not possible to pool the data on the two varieties and consider them as replicates. Although the conditions in Tests 1 and 2 were not alike (Table XVI), the average differences in response to dosage and time of exposure were statistically significant. The analysis of the data in Table XVI is summarized in Table XVII.

There is a highly significant or significant relationship between the dosages as well as the number of exposures used and the extent of tip burn in both varieties, although it is obvious that a given treatment caused about twice as much tip burn in Snow Princess as in Elizabeth the Queen. The only significance between number of times exposed and F content was

TABLE XVI

EFFECT OF TWO EXPOSURES TO HF ON TIP BURN AND F CONTENT OF 4-INCH LEAF TIPS AND DRY WEIGHT OF TOPS OF TWO VARIETIES OF GLADIOLUS HARVESTED AT DIFFERENT TIMES AFTER THE FIRST FUMIGATION (1958)

Variety	Av. concn. HF, p.p.b., at each exposure		F in leaf tips, days after first exposure				Tip burn and dry weight, days after first exposure			
	First exposure	Second exposure	P.p.m.				Tip burn, cm.*			
Test 1	July 23–30	Aug. 12–19	8	28	37	42	8	28	37	42
Snow Princess	<0.05	<0.05	13	10	22	31	1.1	2.0	1.7	2.4
	0.82	0.67	36	23	42	40	6.1	9.2	9.9	9.9
	2.84	2.03	76	39	36	43	18.0	27.0	23.0	29.0
Elizabeth the Queen	<0.05	<0.05	22	25	39	44	0.9	2.1	1.8	2.0
	0.82	0.67	55	58	75	79	3.9	6.9	6.0	5.8
	2.84	2.03	134	166	173	205	9.7	14.0	11.0	15.0
Test 2	July 29–Aug. 6	Aug. 19–25	7	21	26	29	7	21	26	29
Snow Princess	<0.05	<0.05	34	43	36	38	1.6	2.6	2.8	3.0
	0.67	0.81	42	57	55	73	7.6	12.0	14.0	12.0
	2.73	2.70	104	164	110	115	29.0	41.0	42.0	39.0
Elizabeth the Queen	<0.05	<0.05	27	64	50	83	1.2	1.9	2.9	2.9
	0.67	0.81	74	152	105	141	4.8	3.8	11.0	7.3
	2.73	2.70	154	327	280	426	13.0	22.0	24.0	22.0
			μG. F				Dry weight of tops, g.			
Test 1	July 23–30	Aug. 12–19	8	28	37	42	8	28	37	42
Snow Princess	<0.05	<0.05	99	146	277	495	7.5	14.0	17.0	16.0
	0.82	0.67	256	383	821	700	7.0	17.0	19.5	17.5
	2.84	2.03	180	291	359	387	5.0	7.5	8.5	7.5
Elizabeth the Queen	<0.05	<0.05	173	406	980	737	7.5	16.0	25.0	16.5
	0.82	0.67	463	750	1,446	1,330	8.5	13.0	18.5	17.0
	2.84	2.03	1,019	2,246	2,001	3,416	7.5	13.5	11.5	16.5
Test 2	July 29–Aug. 6	Aug. 19–25	7	21	26	29	7	21	26	29
Snow Princess	<0.05	<0.05	233	653	519	654	7.0	15.5	15.0	11.0
	0.67	0.81	378	782	899	1,177	9.0	13.5	16.5	16.0
	2.73	2.70	731	1,535	1,173	982	7.0	9.5	10.5	8.5
Elizabeth the Queen	<0.05	<0.05	267	1,304	750	1,203	10.0	25.0	15.5	14.5
	0.67	0.81	717	2,750	1,976	2,160	10.0	23.0	19.0	15.0
	2.73	2.70	1,003	6,858	3,906	8,441	6.5	21.0	14.0	20.0

* Average length on third, fourth, and fifth leaves of four plants.

TABLE XVII

STATISTICAL SIGNIFICANCE OF DATA IN TABLE XVI

Factors to be evaluated	Degree of significance* in			
	Snow Princess		Elizabeth the Queen	
	F content	Tip burn	F content	Tip burn
Individual factors				
Dose (concn. in air)	High	High	High	High
No. of exposures	None	High	Sign.	Sign.
Houses (replicates)	High	High	High	High
Interaction of factors				
Dose × No. of exposures	None	Sign.	None	None
Dose × house	High	High	High	High
No. of exposures × house	None	None	None	None
Significant difference between means of any 6 values (i.e., 1 wk. vs. 3 wks. etc.)	32.8 P.p.m.	3.3 Cm.	64.1 P.p.m.	3.9 Cm.
of any 8 values (i.e., dose 0.05 vs. 0.75 etc.)	30.8 P.p.m.	3.1 Cm.	60.3 P.p.m.	2.2 Cm.

* High = highly significant at odds >100:1 and significant = >20:1.

in Elizabeth the Queen. While the dosages of HF behaved differently in the replicate houses, in general the response from number of exposures was similar in the replicate houses for both varieties.

Since Snow Princess accumulated fluorides during the first exposure more or less in proportion to the dosage available in the air, it must be concluded that either the leaves had lost F after the initial uptake, or treated plants became refractive to subsequent exposure. The former seems to be more probable since the situation appears to be analogous to what was observed in greenhouse experiments on tomatoes, and with milo maize and sweet corn in field plots during the 1959 experiments (Table XVIII).

These peculiar inverse relationships between amount of fluorides accumulated and the severity of tip burn seem to be of more than passing interest. One may justifiably ask whether the more susceptible varieties of gladiolus are injured by small dosages of fluorides during the process of detoxifying, eliminating, or transporting them to other tissues. With very few exceptions during 7 years of experimentation, the more resistant varieties of gladiolus have accumulated the higher concentrations of F. Something of the same order was observed in the studies on response of different species of plants to fluorides. The more susceptible species had relatively less F in their tissues after exposure to HF. Either the more resistant species and varieties have ability to detoxify and store fluorides in some innocuous form so they will not interfere with further uptake of F, or else the susceptible types have some sort of a system for eliminating F.

The dosage response of the two varieties can be determined by calculating the regression curves for tip injury and F content. There was a highly significant regression of tip burn on fluoride content. As shown by the data in Figure 3, there was a progressive increase in injury as fluorides accumulated, but Elizabeth the Queen had a significantly flatter dosage-response curve than Snow Princess. In order to exhibit an equivalent increase in injury, about four times as much F had to be accumulated by Elizabeth the Queen as by Snow Princess.

Examination of the data in Table XVI reveals that an approximate 3-fold increase in dosage of HF in a single application increased tip burn by 3.4 to 4.5 times in Snow Princess and by 3.4 to 3.9 in Elizabeth the Queen. These ratios were not substantially changed by a second exposure when the data were properly corrected for changes in the untreated controls, and the ratio held fairly constant after treatment to the end of the experiment.

TABLE XVIII

AVERAGE PERCENTAGE LOSS OF F FROM ABOVEGROUND PARTS OF POTTED GLADIOLUS AND TOMATO, AND PLOT-GROWN SWEET CORN AND MILO MAIZE ONE WEEK AFTER FUMIGATION WITH HF GAS (1959)

Species fumigated with HF	Per cent decrease, μg. F per plant	
	From leaves*	From aboveground parts
Snow Princess gladiolus (in Marcross corn plots)	49	48
Elizabeth the Queen gladiolus (in Marcross corn plots)	<1	4
Marcross sweet corn	32	21
Snow Princess gladiolus (in Martin's milo maize plots)	51	51
Elizabeth the Queen gladiolus (in Martin's milo maize plots)	9	8
Martin's milo maize	59	46
Bonny Best tomato	35	45

* Ten-centimeter leaf tips of gladiolus, upper half of middle leaves of corn and milo maize, and the four middle leaves of flowering tomato plants.

The ratio of F in tissues exposed to the higher dosages presented an entirely different picture. In Snow Princess a single application of the higher dosage produced 2.7 to 8.2 times as much F in the leaf tips; a second fumigation did not materially change the ratios, even though it did increase the total amount of F; and in the 2-week period following fumigation the ratio decreased sharply. In Elizabeth the Queen the initial fumigation at the high dosage of fluorides produced 2.7 to 3.7 times as much F in the tissues as at the lower dosage. A subsequent exposure further increased the ratio, showing that the higher concentration was still readily taken up and there was no evidence of a decrease in the ratio following treatment.

The aboveground parts of Snow Princess exposed to the higher dosage weighed significantly less within a week after the second exposure than the plants from the controls or low dosage. By the end of the experiments these differences had been accentuated to the point where the treated plants weighed no more than 50 per cent as much as the controls (Table XVI). There was no evidence of a comparable suppression of tissue synthesis in Elizabeth the Queen at the higher dosage or of either variety at the lower dosage. The dosages employed in these experiments apparently were at a suitable level for differentiating between response of varieties and for establishing threshold values for Snow Princess. It should be noted that very conspicuous tip burn (Table XVI) was caused by these treatments, and there can be no allegation of a so-called masked effect because even plants with considerable visible tip burn from exposure to the lower dosage were not significantly retarded. Apparently the tip burn on Snow Princess had to exceed a length of 14 cm. before top development was adversely affected, and Elizabeth the Queen tolerated up to 24 cm. without being retarded significantly.

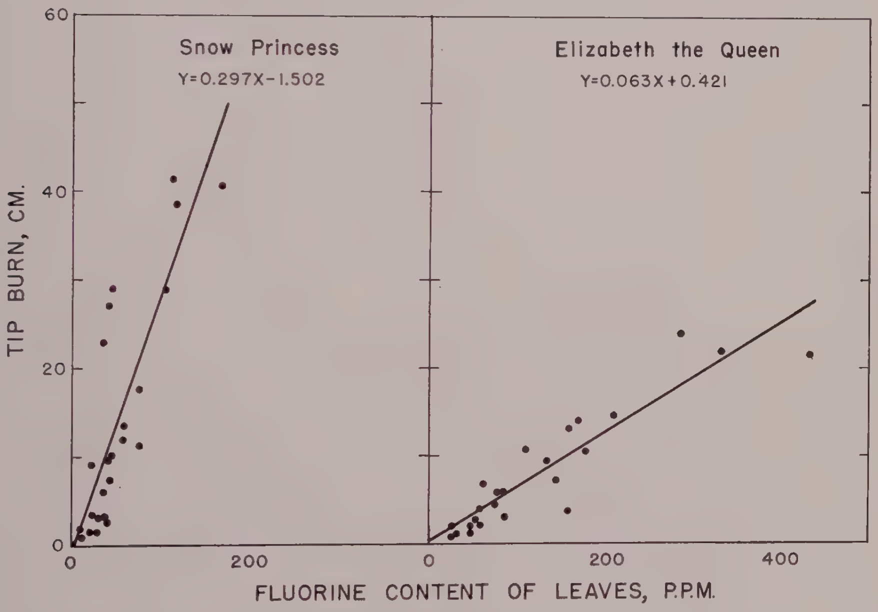

FIGURE 3. Relation of tip burn to F content of gladiolus leaves in the varieties Snow Princess and Elizabeth the Queen.

Effect of gaseous mixtures. The possibility has been considered that toxicity of HF may be synergized by sulfur dioxide (SO_2), hydrocarbons, and other gases known to be present in many localities. In one test five plants of each of four varieties of gladiolus were exposed to a mixture of gaseous hydrocarbons obtained from an oil refinery (exact composition not known), to H_2SiF_6, and to a mixture of these two gases injected separately into the fumigation chamber. The results are shown in Table XIX.

The mixture of hydrocarbons apparently caused no tip burn since there was no more necrosis than in the control. More tip burn was observed on all varieties exposed to H_2SiF_6 than on the controls. The varietal response was essentially the same as to HF since there was a progressive increase in the amount of tip burn in the order listed. The hydrocarbon reduced the severity of the H_2SiF_6 effect, but it should be noted that the mean fluoride content of the air was slightly less than where the fluosilicate was used alone. The differences between the three treatments were highly significant when subjected to statistical analysis.

TABLE XIX

EFFECT OF GASEOUS HYDROCARBON MIXTURE ON TIP BURN OF GLADIOLUS CAUSED BY H_2SiF_6

Variety	Treatment, 38-hour exposure		Severity of tip burn, cm., on leaf No.*					
	Material added to air	Dosage, p.p.b.	5	4	3	2	1	Av.
Elizabeth the Queen	None	0	1	2	1	2	1	1.4
	Hydrocarbon	16	1	2	2	1	0	1.2
	H_2SiF_6	2.7	3	4	4	3	1	3.0
	H_2SiF_6+hydrocarbon	2.4+16	2	3	1	2	1	1.8
Hopman's Glory	None	0	1	2	2	2	1	1.6
	Hydrocarbon	16	0	1	2	2	2	1.4
	H_2SiF_6	2.7	5	10	5	3	2	5.0
	H_2SiF_6	2.4+16	1	1	3	2	2	1.8
Spic and Span	None	0	1	3	2	3	1	2.0
	Hydrocarbon	16	1	2	2	2	1	1.6
	H_2SiF_6	2.7	9	7	5	4	2	5.4
	H_2SiF_6+hydrocarbon	2.4+16	1	4	4	4	2	3.0
Snow Princess	None	0	3	5	4	2	2	3.2
	Hydrocarbon	16	3	4	3	2	4	3.2
	H_2SiF_6	2.7	11	14	9	7	7	9.6
	H_2SiF_6+hydrocarbon	2.4+16	5	4	5	4	7	5.0

* Leaves numbered from No. 1 (oldest) to No. 5 (youngest).

A comparison of the effects of HF and H_2SiF_6 on the variety Snow Princess showed that they induced essentially the same amount of tip burn when used in the dosage range of 3.4 to 8.3 p.p.b. Results with other species showed no synergistic or antagonistic effect between SO_2 and the hydrocarbon mixture or between SO_2 and HF.

Soil fluorides. Tests with varieties Snow Princess and Valeria gladiolus showed that sodium fluoride (NaF) and cryolite (Na_2AlF_6) were taken up from soil in sufficient quantities to increase tip burn and F content of tissue (Table XX). The number of flower spikes and total fresh weight of tops were reduced by applications of the highest level of NaF in variety Snow Princess but not in variety Valeria.

Although the data in Table XX as to F uptake from the soil and type of reaction agree well with observations on exposure to fluorides in the air, varietal reaction can be distinctly different by the two routes of application.

TABLE XX

EFFECT OF ADDITIONS OF FLUORIDE TO SOIL ON GROWTH, YIELD, AND F CONTENT OF TWO VARIETIES OF GLADIOLUS GROWN IN 2-GALLON CROCKS

Soil treatments			Tip burn, % leaf length	No. of flower spikes Aug. 28	Total fresh weight of tops, lb.	F, p.p.m. in 10-cm. leaf tips
Additions to soil surface*	No. of times applied	Total fluoride added, g.				
Variety Snow Princess planted May 27, soil treatments June 26 to August 8, plants harvested August 28, 1957						
Water control	—	None	7	40	9.5	29
0.05% NaF	15	3.0	19	49	10.5	108
0.10% NaF	15	5.6	43	34	6.5	138
Na_2AlF_6	1	6.0	16	38	9.8	58
Variety Valeria planted May 14, soil treatments July 12 to August 9, plants harvested August 28, 1957						
Water control	—	None	9	24	5.8	34
0.05% NaF	12	2.4	14	26	6.0	106
0.10% NaF	12	4.8	15	26	5.9	144
Na_2AlF_6**	1	16.0	20	25	6.0	188

* Six crocks per treatment and four corms planted per crock.

** Mixed with soil at start.

For example, a test made in 1958 showed that Na_2AlF_6 added to the soil at the rate of 6 g. per 2-gal. crock had very little effect on Snow Princess gladiolus but reduced flowering in Elizabeth the Queen by 33 per cent.

NaF added in solution to give 5.6 g. per pot over a period of several weeks reduced the fresh weight of plants, increased the amount of tip burn, and decreased the number of flowering spikes per plant more than Na_2AlF_6. In all three categories its effect on Elizabeth the Queen was more drastic than on Snow Princess. It should be noted that this is exactly the reverse of what was observed repeatedly when these two varieties were exposed to fluorides in the air under controlled conditions. The only point of similarity in the comparative response of the two varieties was the pronouncedly greater accumulation of F by Elizabeth the Queen by both routes of application.

Route of penetration of fluorides into leaf. The exact route of penetration into the leaf has not been determined. The stomata do not appear to be a major factor. Study of representative plants in 1953 failed to show any correlation between the number and location of stomata and the extent of injury. These data were reenforced by studies the following year on 62 species of plants, including gladiolus, which ranged from extremely susceptible to resistant in their response to HF and SO_2 (15). Susceptibility to either of the two gases was not necessarily associated with numerous stomata on either the upper or lower surface of the leaf (15).

Loss of F from leaves and plants. The F taken into the leaf may be stored there or transported elsewhere. There is a very substantial volume of evidence that the F concentration declines one week or more after exposure to HF. The rapid disappearance of F from the foliage may not occur in all species and varieties of plants. However, in gladiolus the variety Snow Princess consistently lost F after exposure. In two experiments the losses were as follows: from the entire tops, 27 to 67 per cent, while controls gained 208 to 293 per cent; from 10-cm. leaf tips, 33 to 69 per cent, while controls gained 200 to 600 per cent; from tops, 40 to 55 per cent, while controls gained 18 to 157 per cent; and from 10-cm. leaf tips, 44 to 64 per cent, while controls gained 7 to 187 per cent. In the same experiments the variety Elizabeth the Queen lost less F from the various plant parts listed above. In the respective plant tissues the changes were 30 to 12 per cent, while controls gained 144 to 145 per cent; 22 to 12 per cent, while controls gained 281 to 263 per cent; 6 to 25 per cent, while controls gained 44 to 59 per cent; and 17 to 2 per cent, while controls were gaining 38 per cent. It is obvious that the very susceptible Snow Princess lost one-third to two-thirds of the F in the tip 10 cm. of leaves and entire plants, while the more resistant Elizabeth the Queen in the same plots showed much smaller changes, rarely exceeding a 20 per cent loss of F from fumigated plants. However, there were enough fluorides in the ambient air (*ca.* 0.35 p.p.b.) to cause the untreated controls of both varieties to gain in F content during the periods of observation. The loss of F from 10-cm. tips and aboveground tops of gladiolus and from leaves and entire tops of milo maize and sweet corn is summarized in Table XVIII. There is sufficient evidence at hand to postulate that each species and possibly each variety has a normal F content of the foliage for a given concentration of fluorides in the air. If the tissue has been overloaded by previous protracted exposure to high concentrations of fluorides, it may lose HF. However, if the same leaf tissue has a low F content, it may pick up F from traces in the air while the other plant is reducing its level. Just what constitutes a normal F equilibrium between tissues and atmosphere cannot be stated, but it would vary directly with the atmospheric level.

Additional data on the accumulation and loss of fluorides by gladiolus

TABLE XXI

CHANGE IN F CONTENT AND DRY WEIGHT OF SNOW PRINCESS GLADIOLUS AFTER TWO EXPOSURES TO HF IN FIELD PLOTS IN LOCATION A

Av. concn. HF, p.p.b.		Shoots per pot					F content of shoots per pot				Per cent change, μg. F
First exposure June 24–July 5	Second exposure July 12–20	Size*	No.		Dry weight, g.**		P.p.m.		μG.		
			July 20	July 28	July 20	July 28	July 20	July 28	July 20	July 28	
Control in ambient air		L	5	5	11.0	13.5	0.39	0.80	4.3	10.8	+151
			5	6	7.5	14.0	0.63	1.6	4.7	22.4	+377
			3	5	9.0	13.1	0.54	0.80	4.9	10.0	+104
		S	5	5	13.0	10.5	1.4	1.3	18.2	13.7	− 25
			5	7	8.8	6.7	0.87	0.78	7.7	5.2	− 32
			5	5	10.5	10.4	1.4	0.71	14.7	7.4	− 50
		Mean			10.0	11.4	0.9	1.0	9.1	11.6	—
Control in washed air		L	4	7	11.3	13.6	0.87	0.81	9.8	11.0	+ 12
			6	4	7.5	12.0	0.56	0.85	4.2	10.2	+143
			7	5	10.4	12.3	0.74	1.0	7.7	12.3	+ 60
		S	8	4	8.2	11.8	1.2	0.80	9.8	9.4	− 4
			5	4	5.8	8.7	1.2	0.42	7.0	3.7	− 47
			5	5	12.3	9.3	0.95	0.55	11.7	5.1	− 56
		Mean			9.3	11.3	0.9	0.8	8.4	8.7	—
1.2	1.2	L	4	8	7.0	10.8	8.8	4.4	61.6	47.5	− 23
			7	8	7.2	14.7	5.1	2.7	36.7	40.0	+ 9
			7	6	10.5	11.2	6.5	3.9	68.3	43.7	− 36
		S	4	8	5.6	9.8	10.3	4.5	58.0	44.1	− 24
			7	5	8.9	10.0	4.8	4.0	42.7	40.0	− 6
			6	6	8.7	7.8	8.0	5.1	69.6	39.8	− 43
		Mean			8.0	10.7	7.3	4.1	56.2	42.5	—
1.6	1.0	L	5	7	8.5	11.1	8.0	3.9	68.0	43.3	− 36
			6	5	6.5	13.5	6.9	5.2	44.9	70.2	+ 56
			5	6	10.0	12.6	11.3	4.3	113.0	54.2	− 52
		S	5	4	6.9	9.8	8.2	5.0	56.6	49.0	− 13
			6	6	8.6	9.6	6.4	3.1	55.0	29.8	− 46
			6	5	8.6	9.0	4.9	4.1	42.1	36.9	− 12
		Mean			8.2	10.9	7.6	4.3	63.3	47.3	—

* One set of pots had larger plants (L) than the other set (S).

** No significant difference between means of controls and HF treatments.

were obtained from experiments carried out in 1960. Results of these tests are shown in Tables XXI to XXIV. It is evident that the accumulation and loss of F in controls and HF-fumigated gladiolus were dependent to a large extent upon the size of the plants. Large Snow Princess plants exposed to ambient air from July 12 to 20 accumulated less F (μg. F per pot) than small plants in Location A (Table XXI) and more in Location B (Table XXIII). During the same period, large control plants in washed air accumulated less F than small plants in both locations. Large control

TABLE XXII

CHANGE IN F CONTENT AND DRY WEIGHT OF ELIZABETH THE QUEEN GLADIOLUS AFTER TWO EXPOSURES TO HF IN FIELD PLOTS IN LOCATION A

Av. concn. HF, p.p.b.			Shoots per pot				F content of shoots per pot				Per cent change, μg. F
First exposure June 24–July 5	Second exposure July 12–20	Size*	No.		Dry weight, g.**		P.p.m.		μG.		
			July 20	July 28	July 20	July 28	July 20	July 28	July 20	July 28	
Control in ambient air		L	5	8	8.0	13.5	1.9	1.4	15.2	18.9	+ 24
			5	8	9.0	17.0	1.4	1.2	12.6	20.4	+ 62
			6	6	8.5	8.0	2.6	2.0	22.1	16.0	− 28
		S	7	5	10.8	6.7	2.0	1.2	21.6	8.0	− 63
			5	6	9.0	7.8	1.4	1.9	12.6	14.8	+ 18
			6	5	8.8	13.3	1.5	1.5	13.2	20.0	+ 52
		Mean			9.0	11.1	1.8	1.5	16.2	16.4	—
Control in washed air		L	7	6	7.9	11.0	1.0	1.5	7.9	16.5	+109
			6	9	9.3	13.5	0.92	0.67	7.4	9.1	+ 23
			6	6	10.5	9.8	1.0	1.0	10.5	9.8	− 7
		S	6	6	7.0	10.3	1.5	1.1	10.5	11.3	+ 8
			6	8	7.3	11.0	1.4	1.0	10.2	11.0	+ 8
			5	6	6.6	9.5	1.4	1.6	9.2	15.2	+ 65
		Mean			8.1	10.9	1.2	1.2	9.3	12.2	—
1.2	1.2	L	6	7	8.5	10.0	14.8	9.0	125.8	90.0	− 28
			7	7	8.7	12.0	10.3	9.0	89.6	108.0	+ 21
			5	8	8.5	9.9	18.4	7.7	156.4	76.2	− 51
		S	5	7	9.5	8.0	7.9	7.5	75.1	60.0	− 20
			7	8	7.5	11.0	9.1	8.7	68.3	95.7	+ 40
			5	5	5.0	12.0	8.2	7.0	41.0	84.0	+105
		Mean			8.0	10.5	11.5	8.2	92.7	85.7	—
1.6	1.0	L	7	7	8.1	11.0	11.3	8.0	91.5	88.0	− 4
			6	7	6.7	11.9	16.2	7.8	108.5	92.8	− 14
			5	6	7.5	8.3	10.3	8.6	77.3	71.4	− 8
		S	5	5	6.0	9.0	7.2	6.3	43.2	56.7	+ 31
			4	7	7.4	12.4	11.8	7.2	87.3	89.3	+ 2
			6	7	7.0	13.6	12.5	5.3	87.5	72.1	− 18
		Mean			7.1	11.1	11.6	7.2	82.6	78.4	—

* One set of pots had larger plants (L) than the other set (S).

** No significant difference between means of controls and HF treatments.

TABLE XXIII

CHANGE IN F CONTENT AND DRY WEIGHT OF SNOW PRINCESS GLADIOLUS AFTER TWO EXPOSURES TO HF IN FIELD PLOTS IN LOCATION B

Av. concn. HF, p.p.b.			Shoots per pot				F content of shoots per pot				Per cent change, µg. F
First exposure June 24–July 5	Second exposure July 12–20	Size*	No.		Dry weight, g.**		P.p.m.		µG.		
			July 20	July 28	July 20	July 28	July 20	July 28	July 20	July 28	
Control in ambient air		L	5	7	12.3	16.8	1.4	1.7	17.2	28.6	+66
			6	8	8.6	16.5	1.0	0.90	8.6	14.9	+73
			8	4	13.3	14.6	0.98	0.84	13.0	12.3	−5
		S	5	5	10.2	11.4	0.63	0.58	6.4	6.6	+3
			7	7	9.0	10.5	1.0	0.66	9.0	6.9	−23
			3	6	7.2	11.0	1.8	1.0	13.0	11.0	−15
		Mean			10.1	13.5	1.1	0.9	11.2	13.4	—
Control in washed air		L	6	6	8.9	12.1	0.90	0.60	8.0	7.3	−9
			5	6	10.5	15.4	0.20	1.0	2.1	15.4	+633
			5	4	9.9	15.0	0.35	1.0	3.5	15.0	+329
		S	7	5	9.2	9.9	1.5	0.51	13.8	5.1	−63
			6	4	10.1	8.0	1.4	0.67	14.1	5.4	−62
			5	4	9.6	9.6	0.90	0.72	8.6	6.9	−20
		Mean			9.7	11.6	0.9	0.8	8.4	9.2	—
1.2	1.1	L	6	6	12.9	16.3	6.8	3.3	87.7	53.8	−39
			7	5	8.0	15.9	4.1	3.4	32.8	54.1	+65
			5	8	9.8	14.2	5.7	2.9	55.9	41.2	−26
		S	5	6	6.8	8.5	7.2	3.7	48.9	31.5	−36
			6	6	9.7	8.6	8.0	4.7	77.6	40.4	−48
			7	6	8.5	10.0	6.7	3.7	57.0	37.0	−35
		Mean			9.3	12.3	6.4	3.6	60.0	43.0	—
1.5	1.3	L	6	5	8.2	15.8	8.8	5.1	72.2	80.6	+12
			5	6	8.5	13.0	5.9	5.4	50.2	70.2	+40
			5	6	7.7	10.3	10.3	6.7	79.3	69.0	−13
		S	7	6	9.3	10.5	9.7	5.1	90.2	53.6	−41
			5	6	7.0	10.5	10.4	5.1	72.8	53.6	−26
			5	8	9.4	10.5	7.3	4.8	68.6	50.4	−26
		Mean			8.4	11.8	8.7	5.4	72.2	62.9	—

* One set of pots had larger plants (L) than the other set (S).
** Required significant difference at 5 per cent between means, 2.7.

Elizabeth the Queen plants accumulated more F than small plants in ambient air and less in washed air in both locations (Tables XXII and XXIV). Eight days after the second fumigation (July 28) large plants of Snow Princess and Elizabeth the Queen exposed to ambient air gained in F (µg. F) by an average of 19 to 211 per cent, whereas small plants of

Snow Princess showed an average loss of 12 to 36 per cent (Tables XXI and XXIII), and small plants of Elizabeth the Queen showed average increases of 2 to 24 per cent (Tables XXII and XXIV).

TABLE XXIV

CHANGE IN F CONTENT AND DRY WEIGHT OF ELIZABETH THE QUEEN GLADIOLUS AFTER TWO EXPOSURES TO HF IN FIELD PLOTS IN LOCATION B

Av. concn. HF, p.p.b.		Shoots per pot				F content of shoots per pot					Per cent change, µg. F
			No.		Dry weight, g.**		P.p.m.		µG.		
First exposure June 24–July 5	Second exposure July 12–20	Size*	July 20	July 28	July 20	July 28	July 20	July 28	July 20	July 28	
Control in ambient air		L	5	6	8.7	14.5	2.6	3.1	22.6	45.0	+ 99
			7	6	12.9	15.5	0.89	1.6	11.5	24.8	+116
			8	7	12.5	11.0	1.3	1.3	16.3	14.3	− 12
		S	5	7	11.0	9.9	1.5	2.0	16.5	19.8	+ 20
			5	7	7.5	13.4	1.7	1.2	12.8	16.1	+ 26
			6	5	7.5	10.6	1.8	1.6	13.5	17.0	+ 26
		Mean			10.0	12.5	1.7	1.8	15.6	22.8	—
Control in washed air		L	5	5	5.8	11.7	0.64	1.7	3.7	19.9	+438
			6	8	11.5	11.6	1.0	2.2	11.5	25.5	+122
			5	8	9.4	8.5	0.61	3.4	7.1	28.9	+307
		S	6	5	10.0	10.7	1.3	1.9	13.0	20.3	+ 56
			5	7	6.9	7.9	1.4	1.7	9.7	13.4	+ 38
			5	4	6.1	10.5	1.4	0.96	8.5	10.1	+ 19
		Mean			8.3	10.2	1.1	2.0	8.9	19.7	—
1.2	1.1	L	7	8	10.3	13.3	8.0	6.7	82.4	89.1	+ 8
			6	6	12.5	12.2	7.7	6.2	96.3	75.6	− 21
			6	7	7.8	13.8	9.0	5.6	70.2	77.3	+ 10
		S	6	5	7.9	13.0	11.8	5.6	93.2	72.8	− 22
			5	6	7.7	10.9	11.6	6.4	93.2	69.8	− 25
			7	4	7.8	8.6	11.4	6.7	123.1	57.6	− 53
		Mean			9.5	12.0	9.9	6.2	93.1	73.7	—
1.5	1.3	L	8	7	9.3	14.7	13.4	7.5	124.6	110.3	− 11
			6	6	9.7	16.5	12.6	7.4	122.2	122.1	− 0
			5	6	8.3	12.7	18.0	8.4	153.0	106.7	− 30
		S	6	6	8.7	9.0	10.0	6.3	87.0	56.7	− 35
			6	6	7.3	12.0	14.3	9.9	104.4	118.8	+ 14
			6	6	7.5	13.0	11.3	6.6	84.8	85.8	+ 1
		Mean			8.5	13.0	13.3	7.7	112.7	100.1	—

* One set of pots had larger plants (L) than the other set (S).

** No significant difference between means of controls and HF treatments.

TABLE XXV

EFFECT OF HF ON DRY WEIGHT OF SNOW PRINCESS GLADIOLUS AFTER TWO EXPOSURES IN FIELD PLOTS IN LOCATION A

Av. concn. HF, p.p.b.		Shoots per pot				
		Size*	No.		Dry weight, g.**	
First exposure June 17–23	Second exposure July 5–11		July 12	July 22	July 12	July 22
Control in ambient air		L	4	8	10.2	10.0
			4	6	8.1	13.6
			5	5	9.2	14.0
		S	5	8	8.0	11.1
			4	5	8.5	12.2
			5	6	7.0	10.5
		Mean			8.5	11.9
Control in washed air		L	5	5	9.8	13.8
			7	4	12.5	15.0
			4	7	11.0	16.5
		S	5	7	12.5	14.4
			5	6	8.0	11.2
			7	7	6.0	10.8
		Mean			10.0	13.6
1.3	0.9	L	6	6	11.0	13.7
			8	7	10.0	12.5
			5	5	12.6	14.4
		S	6	3	8.5	11.0
			6	7	9.0	8.8
			5	5	7.6	10.7
		Mean			9.8	11.9
1.1	0.86	L	8	7	13.6	14.7
			7	4	12.5	13.9
			6	5	6.9	21.8
		S	7	5	10.7	10.3
			4	7	9.0	14.5
			5	7	10.0	10.5
		Mean			10.5	14.3

* One set of pots had larger plants (L) than the other set (S).

** Required difference for significance at 5 per cent between means, 2.2.

EFFECT OF FLUORIDES ON DRY WEIGHT OF TOPS

The tops were collected from Snow Princess and Elizabeth the Queen in pots held in field plots at the same time as the vegetable crops were harvested. There was no evidence that the growth of plants had been adversely affected by holding them under the fumigation houses for two periods of a week each since the controls held in washed air produced just as much dry matter as plants in ambient air throughout the experiment.

There was no significant difference in top development of plants exposed to two dosages of HF and the controls in Locations A and B Tables XXV, XXVI, and XXVII). The plants had been sorted into large and small groups which were distributed as equally as possible in the four

TABLE XXVI

EFFECT OF HF ON DRY WEIGHT OF ELIZABETH THE QUEEN GLADIOLUS AFTER TWO EXPOSURES IN FIELD PLOTS IN LOCATION A

Av. concn. HF, p.p.b.		Shoots per pot				
First exposure June 17–23	Second exposure July 5–11	Size*	No.		Dry weight, g.**	
			July 12	July 22	July 12	July 22
Control in ambient air		L	9	7	8.5	13.2
			5	6	10.0	9.0
			6	8	8.8	12.7
		S	7	7	9.8	8.2
			6	6	7.0	9.2
			5	5	7.4	7.7
		Mean			8.6	10.0
Control in washed air		L	7	7	10.8	17.0
			7	5	10.0	13.1
			5	7	10.0	13.3
		S	5	6	12.5	9.8
			6	5	12.0	10.8
			5	5	10.7	11.5
		Mean			11.0	12.6
1.3	0.9	L	7	9	10.0	14.9
			7	7	11.0	12.8
			7	7	12.7	13.0
		S	6	6	10.8	10.2
			5	5	12.2	12.5
			5	6	9.0	4.5
		Mean			11.0	11.3
1.1	0.86	L	5	8	10.0	14.0
			5	7	11.4	14.5
			6	7	13.0	12.7
		S	6	6	12.0	11.2
			6	5	9.0	9.1
			6	4	13.0	10.5
		Mean			11.4	12.0

* One set of pots had larger plants (L) than the other set (S).

** Required difference for significance at 5 per cent between means, 4.0.

TABLE XXVII

Change in F Content and Dry Weight of Gladiolus after Two Exposures to HF in Field Plots in Location B

Av. concn. HF, p.p.b.		Shoots per pot				F content of shoots per pot				Per cent change, μg. F
First exposure June 17–23	Second exposure July 5–11	No.		Dry weight, g.*		P.p.m.		μG.		
		July 12	July 22	July 12	July 22	July 12	July 22	July 12	July 22	
					Snow Princess					
Control in ambient air		5	6	10.0	12.9	0.20	2.0	2.0	25.8	+1,190
		6	6	13.2	17.5	0.20	1.4	2.6	24.5	+ 842
		5	5	11.0	10.8	0.37	2.1	4.1	22.7	+ 454
		Mean		11.4	13.7	0.26	1.8	2.9	24.3	—
Control in washed air		5	5	13.5	13.9	0.46	0.61	6.2	8.5	+ 37
		8	7	11.0	13.8	0.40	0.78	4.4	10.8	+ 146
		4	7	11.0	11.0	0.62	0.67	6.8	7.4	+ 9
		Mean		11.8	12.9	0.49	0.69	5.8	8.9	—
2.1	1.5	5	5	10.0	13.0	8.1	3.1	81.0	40.3	− 50
		5	6	10.0	14.3	5.8	7.1	58.0	101.5	+ 75
		4	6	12.5	13.1	6.3	6.8	78.8	81.1	+ 3
		Mean		10.8	13.5	6.7	5.7	72.6	74.3	—
2.1	2.1	5	6	10.5	13.3	11.6	8.0	121.8	106.4	− 13
		7	5	10.9	7.5	8.2	2.8	89.4	21.0	− 77
		6	7	14.0	14.2	8.6	8.3	120.4	117.9	− 2
		Mean		11.8	11.7	9.5	6.4	110.5	81.8	—
					Elizabeth the Queen					
Control in ambient air		6	7	10.9	11.6	1.6	3.7	17.4	42.9	+ 147
		4	7	12.5	13.0	1.6	2.1	20.0	27.3	+ 37
		5	7	10.0	11.8	1.7	2.1	17.0	24.8	+ 46
		Mean		11.1	12.1	1.6	2.6	18.1	31.7	—
Control in washed air		7	6	9.8	10.9	0.88	1.3	8.6	14.2	+ 65
		6	6	13.8	13.2	1.0	0.98	13.8	12.9	− 6
		5	7	12.5	14.5	0.93	1.6	11.6	23.2	+ 100
		Mean		12.0	12.9	0.94	1.3	11.3	16.8	—
2.1	1.5	6	9	9.9	16.7	11.5	9.3	113.9	155.3	+ 36
		7	5	12.5	12.4	13.5	6.0	168.8	74.4	− 56
		7	6	16.7	11.5	8.9	11.1	148.6	127.7	− 14
		Mean		13.0	13.5	11.3	8.8	143.8	119.1	—
2.1	2.1	7	5	10.0	8.5	19.4	16.3	194.0	138.6	− 29
		6	6	10.5	10.5	12.5	12.7	131.3	133.4	+ 2
		7	7	14.8	10.8	12.9	13.8	190.9	149.0	− 22
		Mean		11.8	9.9	14.9	14.3	172.0	140.0	—

* No significant difference between means of dry weights for either variety.

treatment lots. The larger plants had significantly heavier tops at the end of the experiment than the smaller ones, but there was no evidence of suppressed growth in either the large or small plants fumigated with HF. Comparable data were obtained on both varieties in another series of tests (Tables XXI, XXII, XXIII, and XXIV). Neither was there a consistent difference between the number of shoots per pot and the dry weight of the plants, as shown by the data on gladiolus in both series of tests (Tables XXI, XXII, XXIII, XXIV, XXV, XXVI, and XXVII). Thus, shoot size was a more important factor than number of shoots per pot in determining the total vegetative growth during the time of these tests.

YIELD OF CORMS FROM FUMIGATED PLANTS

Although numerous experiments have been made with gladiolus, most of these were not continued until corm development was completed. In 1951 five varieties of gladiolus were exposed to HF at concentrations varying from 10 to 32 p.p.b. for five periods of 2 days each. The data for average yield of corms from these treated and control plots summarized in Table XXVIII show that yields were suppressed about 40 per cent by the high dosages of 32 p.p.b. but were not affected at 10 p.p.b. The leaves on the fumigated plants had extensive tip burn.

In one series of tests the effect of low levels of HF (<2 p.p.b.) on the yield of gladiolus corms was determined by the exposure of potted Snow Princess and Elizabeth the Queen (planted May 23) to HF gas in Vinylite covered houses in Location A and in polyethylene covered houses in Location B. The gladiolus were placed in plots of Martin's milo maize during fumigation and alongside the plots thereafter until September 12 when the corms were harvested. The average concentration of HF during the two exposures was nearly the same for each dosage level in Locations A

TABLE XXVIII

YIELD OF CORMS FROM FIVE VARIETIES OF PLOT-GROWN GLADIOLUS EXPOSED TO HF FOR FIVE PERIODS OF TWO DAYS EACH

Variety	Mean weight of corms, g.*			Mean for varieties
	Control	HF treated		
		10 P.p.b.	32 P.p.b.	
Commander Koehl	16.8	21.5	13.0	17.0
Abu Hassan	35.0	32.5	22.5	31.2
Yellow Emperor	45.0	50.5	25.5	41.5
Elizabeth the Queen	36.0	28.5	14.0	28.6
Stoplight	14.8	17.0	13.0	14.9
Mean	29.5	30.0	17.6	

* Required difference for significance at 5 per cent between means of treatment for all varieties, 9.8; for means of varieties, 15.2.

TABLE XXIX

EFFECT OF TWO EXPOSURES TO HF ON YIELD OF CORMS FROM TWO VARIETIES OF GLADIOLUS FUMIGATED IN FIELD PLOTS IN TWO LOCATIONS. (SEE TABLE XXXII FOR NUMBER OF PLANTS WHICH FLOWERED AND SIZE OF CORMS PRODUCED)

Av. concn. HF, p.p.b.				No. of corms				Weight of corms, g.				
First exposure July 20–26		Second exposure Aug. 3–11		Location A		Location B	Total	Location A		Location B	Total	Mean corm weight
Location		Location		Large plants	Small plants	Large plants		Large plants	Small plants	Large plants		
A	B	A	B									
Total yield of corms, Snow Princess												
Control ambient air				30	31	33	94	494	516	440	1,440	15.4
Control washed air				31	28	32	91	521	493	461	1,475	16.2
0.82	0.86	0.72	1.05	35	33	31	99	501	486	388	1,375	13.9
1.21	1.00	1.04	1.16	30	36	34	100	485	483	403	1,371	13.7
Mean				32	32	33	96	500	495	423	1,418	14.8
Total yield of corms, Elizabeth the Queen												
Control ambient air				42	35	39	116	539	547	555	1,641	14.1
Control washed air				37	37	41	115	587	600	524	1,711	14.9
0.82	0.86	0.72	1.05	35	35	44	114	644	617	523	1,784	15.7
1.21	1.00	1.04	1.16	40	36	42	118	617	583	586	1,786	15.1
Mean				39	36	42	116	597	587	547	1,731	14.9
Corms greater than $1\frac{3}{16}$ inch diameter, Snow Princess*												
Control ambient air				23	25	25	73	443	475	395	1,313	18.0
Control washed air				22	22	22	66	452	452	402	1,306	19.8
0.82	0.86	0.72	1.05	26	22	21	69	444	405	337	1,186	17.2
1.21	1.00	1.04	1.16	25	23	17	65	459	408	297	1,164	17.9
Mean				24	23	21	68	450	436	358	1,242	18.2
Corms greater than $1\frac{3}{16}$ inch diameter, Elizabeth the Queen*												
Control ambient air				28	27	31	86	467	483	401	1,351	15.7
Control washed air				28	30	27	85	542	565	408	1,515	17.8
0.82	0.86	0.72	1.05	32	27	30	89	630	567	316	1,513	17.0
1.21	1.00	1.04	1.16	30	28	35	93	556	536	425	1,517	16.3
Mean				30	28	31	88	549	538	388	1,474	16.7

* Jumbo, No. 1, and No. 2 corms.

and B (Table XXIX). Controls consisted of plants exposed to ambient air and washed air. The 12 pots of each variety used per treatment had been divided into two groups of 6 pots each according to the relative size of the shoots (large vs. small). The shoots of small plants in Location B were used for biochemical studies, and hence results on corm yield are not available for this group of plants. After exposure to HF in maize plots,

TABLE XXX

EFFECT OF TWO EXPOSURES TO HF ON THE SIZE AND MEAN WEIGHT OF CORMS PRODUCED BY TWO VARIETIES OF GLADIOLUS FUMIGATED IN FIELD PLOTS IN TWO LOCATIONS

Av. concn. HF, p.p.b.				Per cent corms of different size and mean corm weight, g., for all treatments					Mean corm weight, g.	Per cent corms of different size and mean corm weight, g., for all treatments					Mean corm weight, g.
First exposure July 20–26		Second exposure Aug. 3–11		Jumbo	No. 1	No. 2	No. 3	No. 4		Jumbo	No. 1	No. 2	No. 3	No. 4	
Location		Location		Snow Princess						Elizabeth the Queen					
A	B	A	B												
Large plants, Vinylite covered houses, Location A															
Control ambient air				20	37	20	23	0	16.5	5	43	19	12	21	12.8
Control washed air				19	36	16	29	0	16.8	16	46	14	16	8	15.9
0.82	0.86	0.72	1.05	9	29	37	11	14	14.4	11	66	14	3	6	18.4
1.21	1.00	1.04	1.16	17	33	33	13	3	16.2	13	45	18	15	10	15.4
Mean % Mean weight				16 27.6	34 19.7	27 12.2	19 7.5	7 3.2	16.0 —	11 28.4	50 18.4	16 12.3	12 7.1	11 3.6	15.6 —
Small plants, Vinylite covered houses, Location A															
Control ambient air				19	35	26	16	3	16.7	3	54	20	17	6	15.6
Control washed air				18	43	18	11	11	17.8	14	51	16	8	11	16.2
0.82	0.86	0.72	1.05	12	45	9	24	9	14.7	20	51	6	17	6	17.6
1.21	1.00	1.04	1.16	6	33	25	22	14	13.4	17	44	17	14	8	16.2
Mean % Mean weight				14 27.6	39 19.6	20 11.8	18 7.5	9 4.8	15.7 —	14 28.4	50 18.7	14 12.7	14 8.2	8 3.3	16.4 —
Large plants, polyethylene covered houses, Location B															
Control ambient air				3	36	36	15	9	13.3	3	49	28	13	8	14.2
Control washed air				19	25	25	16	15	14.4	2	46	17	15	20	12.8
0.82	0.86	0.72	1.05	3	42	23	13	19	12.5	5	30	34	7	25	11.9
1.21	1.00	1.04	1.16	9	21	21	29	20	11.9	5	45	33	17	0	14.0
Mean % Mean weight				9 26.7	31 17.6	26 12.7	18 7.4	16 4.0	13.0 —	4 25.3	43 17.6	28 12.5	13 6.6	13 3.5	13.2 —

Snow Princess developed 3.8 to 9.6 cm. of tip burn and Elizabeth the Queen 3.0 to 7.6 cm., depending on concentration.

Neither the number of corms nor the total yield was affected significantly by the fumigation (Table XXIX). However, it should be noted that the susceptible Snow Princess had a consistent tendency to produce somewhat smaller corms and less total weight after fumigation both in regard to total corms and corms over $1\frac{3}{16}$ inches in diameter. There was no evidence of such differences in the resistant Elizabeth the Queen, so these apparent reductions in size must be noted even though the differences were not statistically significant.

There were varietal differences in corm yield and differences due to cultural site. For example, the total number of corms produced by Elizabeth the Queen from large plants in Location A was 35 to 42 (av. 38.5) as

TABLE XXXI

EFFECT OF TWO EXPOSURES TO HF JULY 20 TO 26 AND AUGUST 3 TO 11 ON THE NUMBER, SIZE, AND WEIGHT OF CORMS FROM TWO VARIETIES OF GLADIOLUS FUMIGATED IN FIELD PLOTS IN TWO LOCATIONS

Av. concn. HF, p.p.b., during 2 exposures at 2 locations	New corm size	No., size, and air dry weight of new corms according to concn. of HF and variety of gladiolus and culture site							
		Snow Princess				Elizabeth the Queen			
		Location A*		Location B**		Location A*		Location B**	
		No.	Weight, g.	No.	Weight, g.	No.	Weight, g.	No.	Weight, g.
Control in ambient air	Jumbo	12	325	1	26	3	70	1	24
	No. 1	22	418	12	211	37	683	19	338
	No. 2	14	175	12	158	21	255	11	142
	No. 3	12	88	5	34	5	39	5	41
	No. 4	1	4	3	11	11	37	3	10
	Total	61	1,010	33	440	77	1,084	39	555
Control in washed air <0.05	Jumbo	10	303	6	160	11	319	1	27
	No. 1	24	488	8	140	32	581	19	340
	No. 2	10	117	8	102	8	95	7	86
	No. 3	12	100	5	37	3	22	6	42
	No. 4	3	10	5	22	8	26	8	29
	Total	59	1,018	32	461	62	1,043	41	524
Location A First 0.82 Second 0.72 Location B First 0.86 Second 1.05	Jumbo	7	174	1	28	11	312	2	49
	No. 1	25	478	13	227	41	797	13	223
	No. 2	16	197	7	82	10	115	15	185
	No. 3	12	94	4	27	6	42	3	27
	No. 4	8	44	6	24	4	15	11	39
	Total	68	987	31	388	72	1,281	44	523
Location A First 1.21 Second 1.04 Location B First 1.00 Second 1.16	Jumbo	7	197	3	79	11	311	2	52
	No. 1	22	445	7	126	34	639	19	331
	No. 2	19	225	7	92	16	187	14	175
	No. 3	12	78	10	79	6	44	7	28
	No. 4	6	23	7	27	9	36	0	0
	Total	66	968	34	403	76	1,217	42	586

* Combined yield from large and small plants, 12 pots per treatment.
** Yield for large plants, 6 pots per treatment.

compared with 30 to 35 (av. 31.5) for Snow Princess. The corresponding number of corms larger than $1\frac{3}{16}$ inch diameter was 28 to 32 (av. 29.5) and 22 to 26 (av. 24.0), respectively, for the two varieties. Corm production from large plants in Location B showed similar differences in favor of Elizabeth the Queen.

The corms from these three experiments were classified as to size and each group was weighed. The data on percentage distribution in the five classes and the mean corm weight for each class and each treatment are

TABLE XXXII

EFFECT OF TWO EXPOSURES TO HF ON THE PRODUCTION OF FLOWER SPIKES AND NEW CORMS BY TWO VARIETIES OF GLADIOLUS FUMIGATED IN FIELD PLOTS IN TWO LOCATIONS. (SEE TABLE XXIX FOR TOTAL YIELD OF CORMS FROM ALL PLANTS)

Av. concn. HF, p.p.b.		Size of corms harvested*	No. of plants which flowered, classified according to size of corm								
			Snow Princess				Elizabeth the Queen				Total two varieties**
			Location A		Location B	Total	Location A		Location B	Total	
First exposure July 20–26	Second exposure Aug. 3–11		Large plants	Small plants	Large plants		Large plants	Small plants	Large plants		
Control in ambient air		Jumbo	4	5	1	10	1	0	0	1	11
		No. 1	5	5	5	15	12	10	14	36	51
		No. 2	2	2	7	11	5	4	6	15	26
		Total	11	12	13	36	18	14	20	52	88
Control in washed air		Jumbo	4	3	4	11	5	4	0	9	20
		No. 1	7	7	5	19	7	12	11	30	49
		No. 2	2	2	1	5	3	0	3	6	11
		Total	13	12	10	35	15	16	14	45	80
Location A 0.82	0.72	Jumbo	1	3	1	5	4	3	1	8	13
		No. 1	6	6	4	16	11	6	9	26	42
Location B 0.86	1.05	No. 2	2	1	1	4	1	0	8	9	13
		Total	9	10	6	25	16	9	18	43	68
Location A 1.21	1.04	Jumbo	0	2	3	5	3	5	0	8	13
		No. 1	5	5	4	14	5	4	10	19	33
Location B 1.00	1.16	No. 2	0	0	5	5	1	1	5	7	12
		Total	5	7	12	24	9	10	15	34	58

* Diameter in inches: Jumbo, >2; No. 1, 1.5 to 2; No. 2, 1.25 to 1.5.

** Highly significant difference between number of flowering plants for control and HF fumigated.

set forth in Table XXX. There was a moderate but inconclusive tendency of Snow Princess to produce fewer jumbo corms in the fumigated lots than in the two controls, and the mean weight per corm was slightly less. Elizabeth the Queen showed no comparable difference either as to class distribution or mean weight of corms in the four treatments. Large plants of both varieties produced fewer jumbo and No. 1 corms in Location B.

In order to simplify these data further, the treatment totals of the pooled data for large and small plants are set forth in Table XXXI. These data show that there was a significant effect of location in the field on the size of corms produced, but the differences between treatments were not statistically significant even with this treatment of the data. Both varieties produced fewer jumbo and No. 1 corms in Location B.

Records were taken on the number of flower stalks in each treatment so this could be correlated with size of corm produced subsequently. The data on flowering presented in Table XXXII show that for both varieties the two control lots produced more flower stalks than the two fumigated lots, and the difference was highly significant. In Snow Princess the flowering in the fumigated lots was about 70 per cent of the controls, while in Elizabeth the Queen it was about 80 per cent.

A second experiment on the effects of fluoride on corm size was made with potted gladiolus planted in July. Data on the extent of tip burn November 7 and size distribution of the corms harvested November 14 are presented in Table XXXIII. The data confirm those of the preceding experiment in showing a trend for Snow Princess to produce fewer of the larger size corm (No. 1) after exposure to fluorides than did the controls. Although this was true in both locations, the differences were not significant at the 5 per cent level.

TABLE XXXIII

EFFECT OF HF ON TIP BURN AND SIZE DISTRIBUTION OF CORMS PRODUCED BY TWO VARIETIES OF GLADIOLUS

Av. concn. HF, p.p.b.		Yield of corms of each size, %*											
		Snow Princess**						Elizabeth the Queen					
		No. of corms	Percentage of corms				Av. length tip burn, cm.	No. of corms	Percentage of corms				Av. length tip burn, cm.
First exposure Aug. 11–18	Second exposure Aug. 25–31		No. 1	No. 2	No. 3	No. 4			No. 1	No. 2	No. 3	No. 4	
Fumigated in Vinylite covered houses, Location A													
Control ambient air		35	37	31	14	17	—	35	43	31	20	6	—
Control washed air		31	42	29	23	6	0.7	38	32	45	13	11	0.8
1.04	1.08	29	24	38	28	10	3.3	34	44	32	9	15	3.3
1.29	1.41	39	26	31	21	23	4.8	35	43	26	17	14	3.9
Fumigated in polyethylene covered houses, Location B													
Control ambient air		35	34	29	29	9	—	35	49	34	11	6	—
Control washed air		33	18	42	39	0	0.5	39	44	31	23	3	0.4
0.88	0.95	34	32	24	26	18	3.4	38	24	53	16	8	2.0
1.13	0.86	32	25	34	25	16	3.4	39	49	33	10	8	3.1

* All pots contained more than four plants.
** Plants subjected to second fumigation only.

In the third test relating to the yield of gladiolus corms, levels of HF were used which induced 10 to 14.5 cm. of tip burn on Snow Princess and 4.8 to 10.6 cm. on Elizabeth the Queen (Table XXXIV) which is considerably more than occurred in the two preceding tests (3.8 to 5.0 cm.). Results in Table XXXIV show that HF had a significant effect on the distribution of corm sizes, even though there was no relation between amount of tip burn and size of corm produced by the same plants. HF-fumigated plants produced fewer No. 1 and more No. 3 corms than the controls. The effect of HF on the percentage of size No. 1 corms produced by Snow Princess and Elizabeth the Queen is shown by the results in Table XXXV. The percentages for Snow Princess exposed to the lower and higher concentrations of HF were, respectively, 28 and 18 per cent as compared to 45 and 55 per cent for controls. The corresponding percentages for HF-fumigated Elizabeth the Queen were 47 and 38 per cent and 66 and 70 per cent for controls. Thus, the effect of HF was more pronounced on Snow Princess than Elizabeth the Queen.

Results in Table XXXV also show that the percentage production of size No. 1 corms by the two selected plants per pot varied greatly according to the total number of shoots per pot and that the differences were greater

TABLE XXXIV

EFFECT OF TWO EXPOSURES TO HF ON LEAF TIP BURN AND SIZE OF GLADIOLUS CORMS DERIVED FROM TWO PRESELECTED PLANTS IN POTS CONTAINING DIFFERENT NUMBERS OF SHOOTS AT THE INITIATION OF FUMIGATION (SEE TABLE XXXV FOR PERCENTAGE DIFFERENCES)

Av. concn. HF, p.p.b.		No. of corms different size				Av. tip burn per plant, cm.			
First exposure Sept. 20–27	Second exposure Oct. 4–11	No. 1	No. 2	No. 3	Total	No. 1	No. 2	No. 3	Av.
				Snow Princess					
Control ambient air		8 5	3 9	2 0	13 14	1.6 1.6	1.4 1.4	1.7 0.0	1.6 1.0
Control washed air		7 10	5 2	3 2	15 14	1.3 1.4	1.8 1.2	1.2 1.0	1.4 1.2
1.0	1.0	7 6	9 7	2 2	18 15	6.9 8.2	8.0 6.3	8.4 1.4	7.8 5.3
1.9	2.4	5 2	7 9	6 5	18 16	10.2 12.9	14.7 10.8	10.1 13.2	11.7 12.3
				Elizabeth the Queen					
Control ambient air		17 13	7 8	0 1	24 22	2.3 2.3	3.0 2.5	0.0 1.6	1.8 2.1
Control washed air		15 12	8 6	1 1	24 19	2.1 2.2	2.5 2.1	2.0 1.4	2.2 1.9
1.0	1.0	14 9	6 9	3 2	23 20	7.4 5.0	6.5 6.7	5.1 4.7	6.3 5.5
1.9	2.4	10 6	10 11	2 2	22 19	9.6 10.3	10.7 8.8	8.4 10.3	9.6 9.8

TABLE XXXV

EFFECT OF TWO EXPOSURES TO HF ON THE YIELD OF NO. 1 GLADIOLUS CORMS FROM TWO PRESELECTED PLANTS IN POTS CONTAINING DIFFERENT NUMBERS OF SHOOTS AT THE INITIATION OF FUMIGATION. RESULTS ARE BASED ON DATA IN TABLE XXXIV

No. of shoots per pot		Percentage of No. 1 corms according to number of shoots per pot and fumigation treatment				
Aug. 11	At start* of fumigation Sept. 20	Controls		Av. concn. HF, p.p.b.		Mean
		Ambient air	Washed air	1.0, 1.0	1.9, 2.4	
		Snow Princess				
>4	5	83	0	29	25	34
>4	4	50	100	0	0	38
4	4	40	83	60	43	57
3	4**	25	67	0	0	23
>4	3	33	50	0	0	21
4	3	50	83	75	40	62
3	3	33	0	33	17	21
Mean		45	55	28	18	—
		Elizabeth the Queen				
>4	5	50	100	0	50	50
>4	4	67	67	50	46	57
4	4	64	45	50	25	46
3	4	100	50	67	50	67
>4	3	75	75	67	33	63
4	3	40	50	67	60	54
3	3	67	100	25	0	48
Mean		66	70	47	38	—

* The number of shoots remained the same or was reduced by cutting off the tops of some plants September 20.

** A new fourth shoot emerged after August 11.

for Snow Princess than for Elizabeth the Queen. For example, HF-fumigated Snow Princess failed to produce any No. 1 corms when the total number of shoots per pot was, respectively, >4, 3, and >4 on August 11, and 4, 4, and 3 at the start of fumigation on September 20. The corresponding production for plants held in ambient and washed air was 50 and 100, 25 and 67, and 33 and 50 per cent No. 1 corms. Thus, the mean percentage yields from these particular control and HF-fumigated Snow Princess plants were among the lowest for all four treatments, as indicated in the last column of Table XXXV, namely, 38, 23, and 21 per cent. The highest mean yields (62 and 57 per cent) were obtained with Snow Princess in pots that had a total of four shoots on August 11 and, respectively, 3 shoots (one shoot cut off) and 4 shoots on September 20. At the start of fumigation flower spikes were starting to emerge on a few plants. In contrast, the yield of No. 1 Elizabeth the Queen corms was not noticeably affected by the total number of shoots per pot.

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COMPARATIVE BIOCHEMISTRY OF OBLIGATELY PARASITIC AND SAPROPHYTIC FUNGI. II. ASSIMILATION OF C^{14}-LABELED SUBSTRATES BY GERMINATING SPORES[1]

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SUMMARY

Comparisons were made of the incorporation of carbon from acetate-2-C^{14}, D-glucose-U-C^{14}, L-leucine-U-C^{14}, and L-glutamate-1-C^{14} into respiratory CO_2, amino acids, organic acids, sugars, nucleic acids, and protein of fungal spores. Uredospores of *Uromyces phaseoli*, *Puccinia helianthi*, and *Puccinia sorghi*, and conidia of *Glomerella cingulata*, *Aspergillus niger*, and *Neurospora sitophila* were employed for these studies. Uredospores utilized less substrate materials than did conidia of saprophytic fungi. Germination of uredospores was not accompanied by net protein or nucleic acid synthesis, as was the case with conidia, and protein turnover was undiminished. About half of each substrate consumed by uredospores was converted to carbon dioxide, while the remainder accumulated in the cellular intermediates. Conidia also converted about half of the assimilated substrate to carbon dioxide, but the rest was largely converted to proteins and nucleic acids. The inhibitors, fluoride, cyanide, fluoroacetate, malonate, and 2,4-dinitrophenol, had only a small effect on uredospore metabolism compared to their effect on conidia, probably reflecting the low rate of synthetic processes in the uredospore. Puromycin enhanced consumption of acetate and glutamate, and depressed glucose assimilation, but did not affect leucine incorporation. The increased synthesis of intermediates by uredospores in the presence of puromycin suggests that such syntheses are ordinarily repressed, possibly as a result of the failure to synthesize protein and nucleic acids.

INTRODUCTION

It is established that uredospores fail to grow in the absence of their living host, yet germinate readily even in distilled water. Host-obligate parasite relations are complicated by extreme host specificity, and a compatible gene-for-gene relationship is necessary for successful parasitism (6). Other plant pathogens, e.g. Fusarium wilts, exhibit equally complex host requirements (26), yet all forms can be cultured on simple media *in vitro*. For these reasons it was thought possible that uredospores also might exhibit less complexity apart from their hosts. Consequently a study of substrate assimilation by various fungi including obligate parasites was started to obtain an insight into the cause of growth failure in uredospores.

Previous studies show that nongerminating uredospores assimilate

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[2] Deceased Feb. 4, 1962.

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acetate at a much slower rate than do nongerminating conidia of saprophytic fungi, but incorporate amino acids into proteins at a rate about equal to that of some saprophytic spores (28). The present report extends these studies to germinating spores.

MATERIALS AND METHODS

Uredospores of *Uromyces phaseoli* (Pers.) Wint., *Puccinia sorghi* Schw., and *Puccinia helianthi* Schw. were obtained from susceptible greenhouse-grown plants. Nearly perfect germination of uredospores was obtained in all trials unless otherwise specified. Radioactive uredospores were obtained from susceptible plants grown in the greenhouse in a partial atmosphere of $C^{14}O_2$. Conidia of *Glomerella cingulata* (Stone.) Spaul. & von Schr. and *Aspergillus niger* van Tiegh were obtained from cultures on potato-dextrose-agar slants kept at 21° C. in the dark, employing the methods of McCallan and Wilcoxon (15). Conidia of *Neurospora sitophila* (Mont.) Shear & Dodge were obtained from mycelia growing on Haurowitz's medium as described by Owens (17). All cultures were 10 days old at harvest and the spores were at least 70 per cent viable.

Uredospores of *U. phaseoli* were stored at 5° C. for no more than 2 weeks after harvest. Uredospores of other species were frozen upon harvest and stored at −20° C. Conidia were utilized immediately after harvest.

Sodium acetate-2-C^{14} and glucose-U-C^{14}, purchased from New England Nuclear Corporation, Boston, Mass., had specific activities of 0.24 μc./mg. and 7.5 μc./mg., respectively. L-Leucine-U-C^{14}, biosynthesized by Schwarz Bioresearch, Inc., Mt. Vernon, N. Y., had a specific activity of 1.1 μc./mg. L-Glutamic acid-1-C^{14}, purchased from California Corporation for Biochemical Research, had a specific activity of 20 μc./mg.

Puromycin was obtained as a gift from Lederle Laboratories, Pearl River, N. Y. and chloromycetin from Parke, Davis and Co., Detroit, Michigan.

PROCEDURE FOR THE GERMINATION OF SPORES

Uredospores. To deplete uredospores of germination inhibitors (1, 8), 500 mg. of dry spores were dispersed over a water surface for 2 hours. The liquid was removed by filtration on a sintered glass filter. Usually 50 mg. of the spores were taken up in 20 ml. 0.1 per cent aqueous Tween 20 by shaking, and then diluted to 200 ml. with water containing the isotope and other substrate materials. Germination was carried out in 1-liter Erlenmeyer flasks on a reciprocating shaker at 20° C. A communication describing the method has appeared elsewhere (32).

Conidia. Conidia were counted by the method of McCallan and Wilcoxon (15), and were suspended in 200 ml. of 0.01 per cent aqueous Tween 20 in liter Erlenmeyer flasks. Titres were held to about 10^6 spores per ml. and germination was carried out on a reciprocating shaker at 20° C.

ANALYTICAL PROCEDURES

The principal analytical procedures have already been described in detail (28). At the termination of the experiments, the spores were extracted with 80 per cent (v/v) ethanol to obtain soluble intermediates. The nucleic acids were extracted with boiling 5 per cent trichloroacetic acid (20), and the residue was hydrolyzed with 6*N* hydrochloric acid to yield a protein amino acid fraction.

The soluble intermediates were separated into cationic (amino acids), anionic (organic acids), and neutral (sugars) fractions using appropriate ion exchange resins. Chromatography and autoradiography of amino acids were carried out as described previously (28). Organic acids were determined by the method of Hulme and Wooltorton (10), and the sugar content of the neutral fraction was examined using a modification of the ion-exchange procedure described by Parr (19).

Protein nitrogen was determined by the micro Kjeldahl procedure (16). Separate aliquots of the protein amino acid fraction were taken for hexosamine determination as described by Owens (18). Ribonucleic acid (RNA) in the hot TCA soluble fraction was determined by the method of Webb (34), and deoxyribonucleic acid (DNA) by the method of Webb and Levy (35).

Radioactivity of the various fractions, column effluents, carbon dioxide, and hydrolyzates was measured on aliquots of the various samples dried on aluminum planchets using a Nuclear Chicago D47 thin-end window gas flow counter.

RESULTS

The response of the germinating fungus spores was studied in terms of: (a) their general assimilation of acetate, (b) their conversion of acetate to various intermediates, (c) protein synthesis, (d) leucine incorporation into protein, and (e) the effect of various inhibitors on substrate consumption. A short study of the physiology of uredospore germination in shake culture is reported, since the method was especially devised for this work.

THE COURSE OF GERMINATION OF UREDOSPORES IN SHAKE CULTURES

Germinating uredospores of *P. sorghi* and *U. phaseoli* from shake cultures were examined microscopically at 2-hour intervals to follow uredospore germination under conditions of the shake culture technique. The data in Figure 1 show that under these conditions germination required 6 to 8 hours for completion. Germ tubes elongated steadily throughout the period, reaching lengths about 15 times the diameter of the spore in 10 hours.

A further study was made of the effects of storage, buffers, and solute concentrations on germination of uredospores. The effects of storage on spores of *U. phaseoli* are shown in Figure 2, where the regression of germination percentage is plotted against storage time. The viability of spores stored at 15° C. rapidly decreased so that by the end of 6 months germination was only about 5 per cent. Uredospores of *P. sorghi* used in the present experiments were kept frozen, in which condition they retain viability for several years, as do uredospores of *P. helianthi* (21).

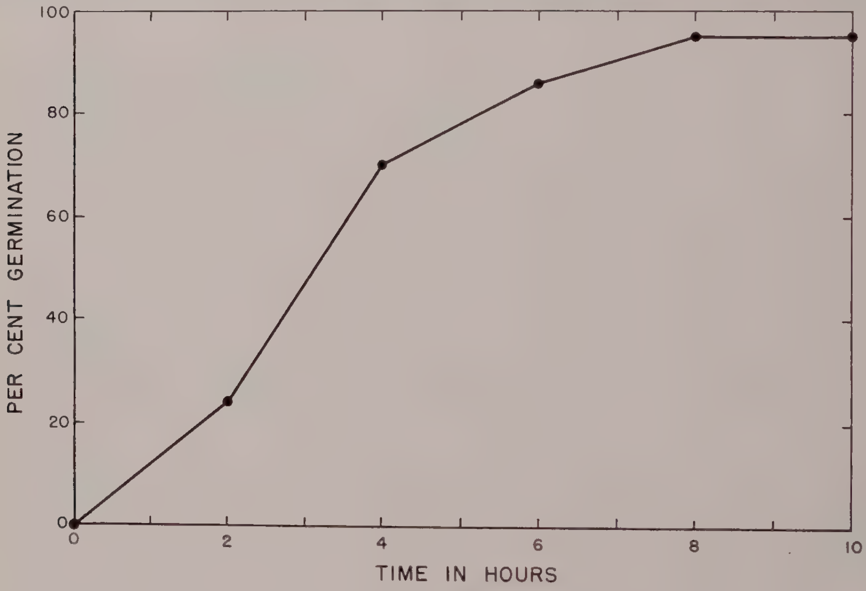

FIGURE 1. Progress of germination of uredospores of *P. sorghi* with time.

The optimum pH for germination of spores of *U. phaseoli* and *P. sorghi* in $10^{-2}M$ phosphate buffer was found to be 6.0 and 4.5, respectively. However, consistently high germination of spores of both species was obtained without buffer, provided substrate concentrations were kept below $10^{-4}M$. Dilute substrate solutions were found to be desirable because the spores generally were inhibited by substrates at concentrations greater than $10^{-2}M$, except for sugars which could be used at concentrations up to $10^{-1}M$. NH^+ at $10^{-4}M$ was toxic to the spores at the optimum pH for germination.

Tween 20 was employed to disperse the spores more readily. Tween 20 was the only wetting agent that was not inhibitory to germination in a long list of such agents tested.

COMPOSITION OF SPORES

The composition of ungerminated uredospores with respect to major constituents has been investigated in detail (27, 28, 30). However, comparable data are not available for germinated spores. Since it is the germinated spores that are concerned in parasitism, it seemed logical to examine the composition and metabolism of uredospores and germ tubes after germination for possible biochemical clues to the cessation of germ tube growth in the absence of a specific host. Uredospores germinate readily *in vitro* as do conidia of saprophytic fungi, except that a transition from spore to mycelium is not accomplished by obligate parasites in the absence of a living host. Consequently comparative studies on several saprophytes were made simultaneously in a search for metabolic differences that might provide a clue to the differences in the ability to grow.

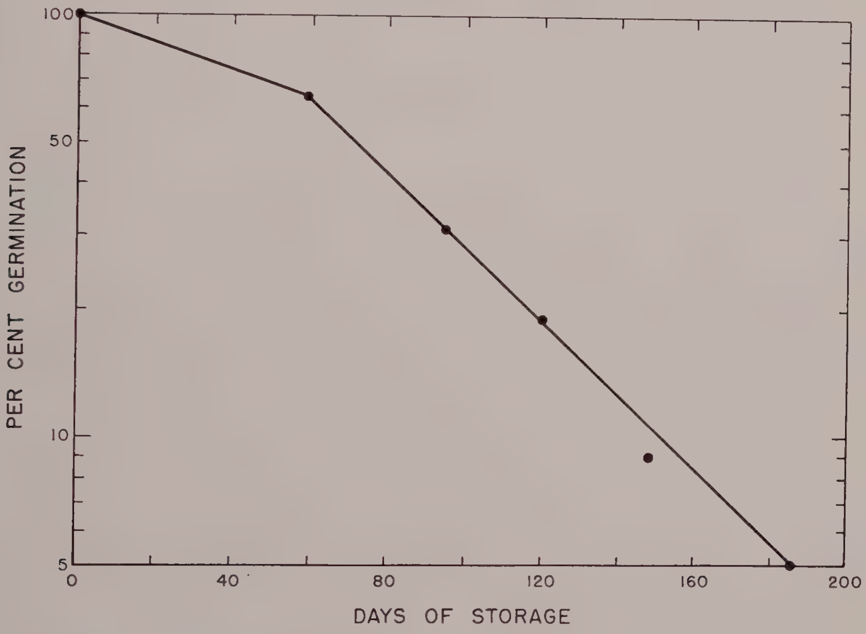

FIGURE 2. Changes in germination of *U. phaseoli* during storage at 15° C.

Uredospores. Usually 95 per cent of the total radioactivity of the amino acid fraction was associated with amino acids. The organic acid fraction consisted predominantly of citric and malonic acids along with minor quantities of other organic acids. If exposure of the spores to radioacetate exceeded 2 hours, citrate contained about 50 per cent of the label. Nearly 45 per cent of the remainder was associated with succinate, although other minor constituents of the Krebs and glyoxylate cycles were also labeled.[3] The major part of the sugar fraction was trehalose with which most of the radioactivity of the fraction after exposure to radioacetate was associated. Minor sugars included glucose, ribose, fructose, and mannose. Mannitol was isolated from bean rust uredospores,[3] but quantitative data concerning the hexitols have yet to be obtained.

The crude protein fraction after hydrolysis consisted largely of amino acids, but about 3 to 15 per cent of the nitrogen and 20 to 40 per cent of the C^{14} occurred as glucosamine-HCl, the amount varying with extent of germination. The radioactivity reported in the protein fraction is corrected for that in glucosamine. Radioactive glucosamine was not detected in nongerminated uredospores (28).

The nucleic acid fraction contained 1.2 mg. RNA per mg. protein nitrogen and 0.07 mg. DNA per mg. protein N. These values did not change with germination.

Conidia. The amino acid and protein fractions from germinating conidia of *A. niger* and *G. cingulata* were composed mainly of C^{14}-amino acids. Glucosamine accounted for less than 1 per cent of the C^{14}, but 3 to 5 per cent of the N in the protein fraction. The organic acid fraction from *A. niger* conidia consisted mainly of Krebs cycle organic acids, particularly citrate. The sugar fraction consisted of sucrose and traces of other sugars.

GENERAL UTILIZATION OF ACETATE

It is possible to express assimilation of acetate in a number of ways (15, 25, 36). Data based on spore weight and initial protein nitrogen indicated that conidia assimilated about 5 times as much substrate as did uredospores, excluding carbon dioxide production from substrate carbon. Values expressed on final protein nitrogen and RNA or DNA indicated the same order of magnitude.

Some of the data concerning gas exchange by germinating spores are summarized in Table I. Carbon dioxide is recorded only as radioactivity since this was directly related to substrate consumption. The data on $C^{14}O_2$ indicate that spores of the different fungi had markedly different capacities to utilize the exogenous substrates. However, the uredospores converted approximately the same proportion of the assimilated substrate to carbon dioxide as did the spores of saprophytic fungi.

The results of a comparative study of acetate-2-C^{14} assimilation by spores of several fungi are summarized in Table II. Protein accumulated with germination of the spores of the saprophytes and there was a parallel consumption of acetate. On the other hand, there was no increase in protein in uredospores upon germination and consumption of acetate remained at a low level. The net synthesis of protein in the saprophytes is

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[3] Staples, Richard C. Unpublished data.

TABLE I

ASSIMILATION OF C^{14} FROM ACETATE BY GERMINATING UREDOSPORES OF THREE SPECIES OF RUST AND CONIDIA OF THREE SAPROPHYTIC FUNGI

Species of fungi	Distribution of C^{14} after 10 hours, c.p.m. $\times 10^{-4}$/mg. of protein N		
	Released as $C^{14}O_2$	Fixed in spore	Ratio $\frac{CO_2}{\text{spore carbon}}$
U. phaseoli	4.4	13.1	0.34
P. helianthi	5.0	19.5	0.26
P. sorghi	5.9	34.2	0.17
G. cingulata	66	206	0.32
A. niger	60	198	0.30
N. sitophila	431	966	0.45

Medium contained 5 µc. of sodium acetate-2-C^{14}, 0.06*M* glucose, and 2% (v/v) of orange juice in distilled water.

reflected also in the distribution of C^{14} from acetate among the various fractions (Table II). Twenty to 60 per cent of the incorporated C^{14} was found in the proteins of the saprophytes whereas only 7 to 9 per cent appeared in the proteins of the rusts. The rusts, on the other hand, accumulated more of the label in sugars and organic acids than did the saprophytes. They also tended to accumulate the label in amino acids, which, in view of the lack of net protein synthesis, suggests a deficiency or block in the protein-synthesizing system of the rusts which drastically lowers the rate of protein synthesis. The nature of the deficiency or block is as yet unknown.

ACETATE ASSIMILATION DURING SPORE GERMINATION

The over-all assimilation of acetate is depicted in Figure 3. Conidia of *A. niger* rapidly synthesized ethanol-soluble intermediates. Radioactivity accumulated in these fractions for 2 hours before the beginning of protein synthesis. Germination was seen after 4 hours. The organic acids, pri-

TABLE II

DISTRIBUTION OF C^{14} AMONG CELL METABOLITES OF UREDOSPORES OF THREE RUST FUNGI AND CONIDIA OF THREE SAPROPHYTIC FUNGI

Species of fungus tested	Protein nitrogen, mg.		C^{14}-assimilated, c.p.m. $\times 10^{4}$ per mg. protein N	Distribution of C^{14} in various fractions, %					
	Initial	Final		Amino acids	Organic acids	Sugars	Nucleic acids	Crude proteins	Glucosamine
U. phaseoli	0.3	0.3	6.3	10	14	40	23	9	4
P. helianthi	0.3	0.3	6.4	13	14	42	21	7	2
P. sorghi	0.4	0.4	28.8	11	19	41	17	7	5
G. cingulata	0.4	0.7	152	14	8	35	23	20	Trace
A. niger	0.4	1.3	140	5	11	26	26	31	Trace
N. sitophila	0.4	1.6	680	3	1	20	16	60	Trace

Spores were germinated for 10 hours in a medium containing 5 µc. of sodium acetate-2-C^{14}, 0.06*M* glucose, and 2% (v/v) of orange juice.

marily citric, began to accumulate in *A. niger* conidia when the rate of growth decreased. Organic acids in uredospores of *U. phaseoli* reached saturation at least by 2 hours.

Figure 4 shows the synthesis of protein by spores of *A. niger* and *U. phaseoli.* Protein content of *A. niger* spores decreased slightly during the first 2 hours of incubation and then increased almost logarithmically for 8 hours. There was no measurable change in the protein content of *U. phaseoli* uredospores, as was demonstrated earlier by Shu, Tanner, and Ledingham for *P. graminis* uredospores (24). The specific activity of *A. niger* protein increased abruptly from an asymptotic value when protein synthesis began. In contrast, the specific activity of uredospore protein remained at a value approaching 3,000 c.p.m. per mg. N.

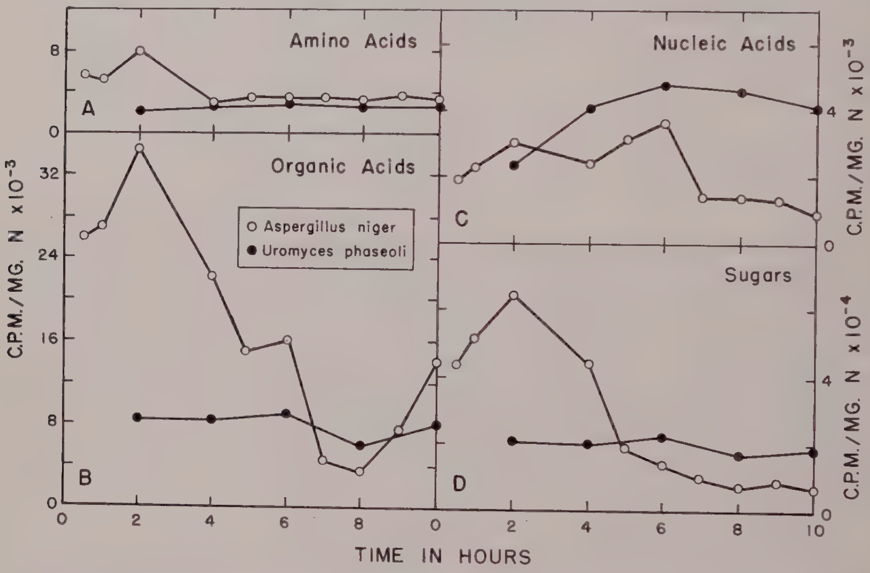

FIGURE 3. The utilization of C^{14}-labeled acetate by fungus spores during germination. Medium included 0.05M glucose, 5 μc. sodium acetate-2-C^{14}, 2% orange juice, and 0.01% Tween 20.

EFFECT OF INHIBITORS ON ACETATE UILIZATION

Uredospores. The effects of several metabolic inhibitors on acetate-2-C^{14} incorporation by uredospores of *U. phaseoli* were determined (Table III). At the concentrations employed, sodium cyanide diminished acetate incorporation about 50 per cent whereas the other compounds caused only slight inhibition, ranging from 7.5 per cent for sodium fluoride to 25 per cent for 2,4-dinitrophenol. In general, none of the compounds caused any drastic change in the distribution of C^{14} from acetate.

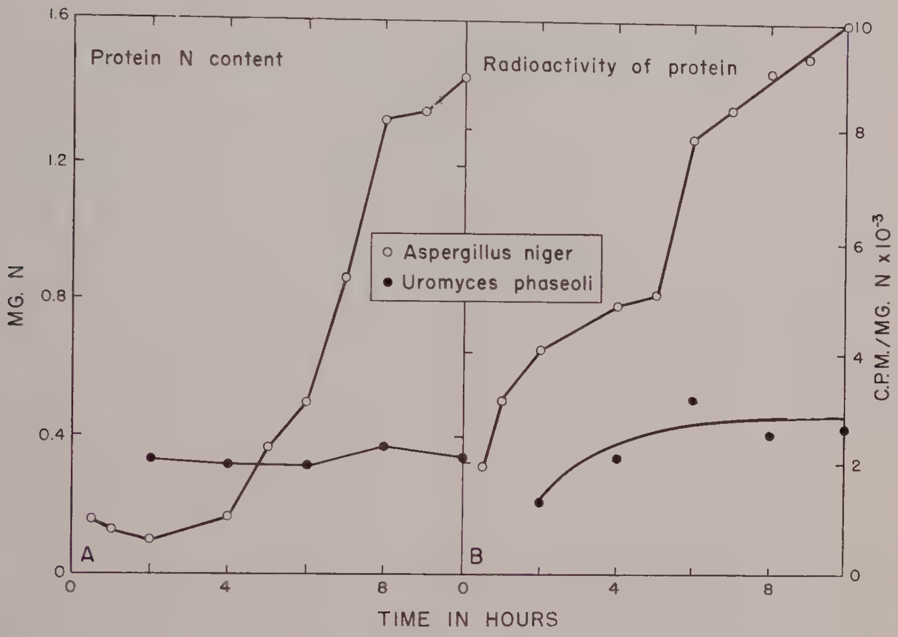

FIGURE 4. Protein synthesis and assimilation of C^{14}-acetate into protein of conidia and uredospores by fungus spores during germination. Medium as in Figure 3.

Further studies utilizing compounds which are known to affect protein synthesis in other organisms (2, 9) were carried out with uredospores of *P. sorghi*. The results are presented in Table IV. Chloromycetin and 3,4-benzpyrene at the concentration used had no effect on germination or C^{14} assimilation and distribution among the various fractions. *p*-Fluorophenylalanine, however, caused about 70 per cent inhibition of germination and a decrease of 35 per cent in acetate assimilation. Both amino and organic acid fractions accumulated label to abnormally high levels

TABLE III

EFFECT OF METABOLIC INHIBITORS ON ACETATE ASSIMILATION BY UREDOSPORES OF U. PHASEOLI

Inhibitor and concentration	C^{14} assimilated, c.p.m. $\times 10^{-4}$ per mg. protein N	Distribution of C^{14} in various fractions, %					
		Amino acids	Organic acids	Sugars	Nucleic acids	Crude proteins	Glucosamine
Control	40	15	11	38	24	8	3
Sodium fluoride, $10^{-2}M$	37	16	13	35	27	5	3
Sodium cyanide, $10^{-3}M$	20	10	20	41	21	4	2
Sodium fluoroacetate, $10^{-3}M$	33	15	11	35	25	8	3
Sodium malonate, $2.5 \times 100^{-2}M$	32	20	8	33	28	5	3
2,4-Dinitrophenol, $1.3 \times 10^{-4}M$	30	13	15	38	24	6	3

Spores were germinated for 10 hours with inhibitor, $0.005M$ glucose, 5 μc. sodium acetate-2-C^{14}, $0.01M$ phosphate buffer, pH 6.2. Each flask contained sufficient spores to yield 1.1 mg. protein nitrogen.

TABLE IV

EFFECT OF PROTEIN INHIBITORS ON ACETATE ASSIMILATION BY UREDOSPORES OF P. SORGHI

Inhibitor and concentration	C^{14} assimilated, c.p.m. $\times 10^{-4}$ per mg. protein N	Germination, %	Distribution of C^{14} in various fractions, %					
			Amino acids	Organic acids	Sugars	Nucleic acids	Crude proteins	Glucosamine
Control	52	99	11	12	39	25	7	5
Chloromycetin, 0.02%	52	98	10	14	33	26	11	6
3,4-Benzpyrene, 0.001%	51	96	12	11	39	25	7	5
p-Fluorophenylalanine, 0.01%	34	28	34	20	31	2	7	4

Spores were germinated 10 hours with $0.005M$ glucose, 5 μc. sodium acetate-2-C^{14}, and $0.01M$ KH_2PO_4. Each flask contained sufficient spores to yield 1.2 mg. protein nitrogen.

whereas very little label appeared in the nucleic acid fraction. The compound had no effect on the labeling of proteins, which supports the suggestion that the proteins of uredospores become labeled by a process of amino acid turnover rather than by synthesis of new protein. Inducible enzymes, such as those reported by van Sumere (31) are undoubtedly fashioned from turnover products.

Conidia. A. niger conidia were poisoned by all the inhibitors used except chloromycetin. There was also a marked reduction in net protein synthesis (Table V) and in the specific activity of the protein fractions. These changes were accompanied by an increase in content of alcohol-soluble intermediates. When the synthesis of protein and nucleic acid was inhibited, intermediates accumulated, but total consumption of substrate was reduced. This effect appears to be similar to that observed in uredospores.

LEUCINE INCORPORATION BY GERMINATING FUNGUS SPORES

Leucine incorporation into protein. The incorporation of L-leucine-U-C^{14} into protein by germinating conidia is shown in Figure 5. Two-dimen-

TABLE V

EFFECT OF INHIBITORS ON ACETATE ASSIMILATION BY CONIDIA OF A. NIGER

Inhibitor and concentration	Protein nitrogen, mg.	C^{14} assimilated, c.p.m. $\times 10^{-4}$ per mg. protein N	Germination, %	Distribution of C^{14} in various fractions, %				
				Amino acids	Organic acids	Sugars	Nucleic acids	Crude proteins
Control	0.9	110	93	4	7	13	21	56
Sodium fluoride, $10^{-3}M$	0.4	78	24	3	8	11	18	60
Sodium cyanide, $10^{-3}M$	0.5	95	1	25	8	9	15	42
Sodium fluoroacetate, $10^{-3}M$	0.5	120	1	9	18	16	13	42
Sodium malonate, $2.5 \times 10^{-2}M$	0.5	150	—	12	5	25	16	40
2,4-Dinitrophenol, $1.3 \times 10^{-4}M$	0.5	100	52	5	8	12	12	62
Chloromycetin, 0.02%	0.9	110	93	5	7	14	15	57

Spores were germinated for 10 hours with inhibitor, 2% (v/v) orange juice, $0.05M$ glucose, 5 μc. sodium acetate-2-C^{14}, and $0.01M$ KH_2PO_4. Initially, 0.4 mg. protein nitrogen was present.

sional chromatograms of protein amino acids showed only leucine to be radioactive after 5 hours. After 8 hours, uredospores of *U. phaseoli* converted about 10 per cent of the C^{14} of leucine into glutamic acid. Conidia of *G. cingulata* rapidly converted leucine to many amino acids and by 8 hours radioactivity in leucine was less than in glutamic acid. Leucine was helpful in following the trend of protein synthesis in *G. cingulata*, but the data were not satisfactory for a rigorous quantitative analysis.

Uredospores of *U. phaseoli* and *P. sorghi* showed no change in rate of protein synthesis during germination and leucine was incorporated at a steady linear rate (Fig. 5). When totally labeled uredospores were incubated with nonradioactive leucine, 35 per cent of the C^{14}-leucine was released in 2 hours. As there is about 1.1 mg. leucine(s) per mg. protein N in *U. phaseoli* uredospores (28) and as the total initial radioactivity was about 1,600 c.p.m. per mg. protein N, the specific activity was approximately 1,450 c.p.m. per mg. leucine. This indicates a loss of 0.4 mg. per hour of leucine since 620 c.p.m. per mg. protein N were lost in 2 hours. This corresponds to an incorporation of 0.3 mg. leucine per hour since leucine was incorporated linearly at a rate of 210,000 c.p.m. per hour and had a specific activity of 671,000 c.p.m. per mg. nitrogen. These results indicated that the rates of incorporation and degradation were similar. The linear loss of radioleucine from uredospore protein confirms the con-

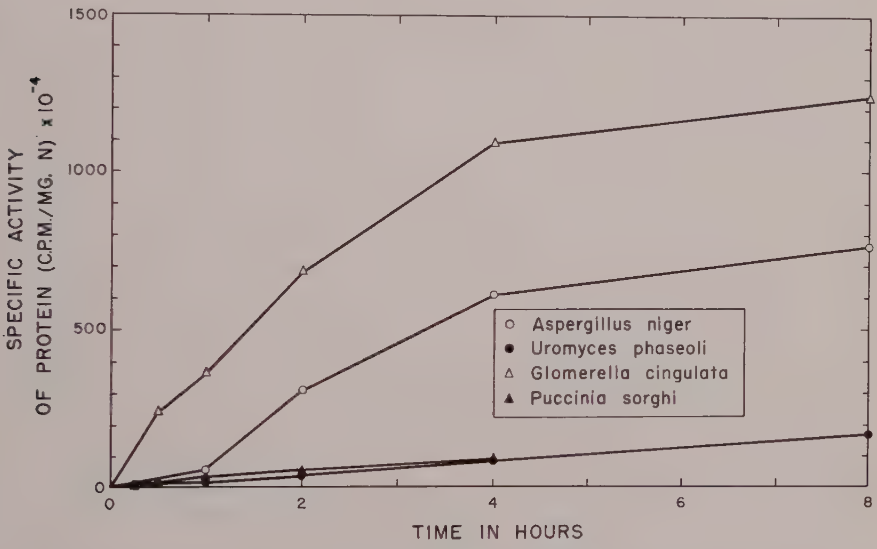

FIGURE 5. Incorporation of C^{14}-labeled leucine into protein by germinating spores of *U. phaseoli, P. sorghi, G. cingulata*, and *A. niger*. Medium included 0.05*M* glucose, 2 μc. L-leucine-U-C^{14}, 2% orange juice, and 0.01% Tween 20.

clusion that protein synthesis occurred in germinating uredospores only under turnover conditions (14). This is in contrast to the results with saprophytic spores in which germination was accompanied by net protein synthesis.

Effect of inhibitors on leucine incorporation into protein. Leucine incorporation into protein of spores of *P. sorghi*, *U. phaseoli*, or *A. niger* in the presence of $10^{-4}M$ 2,4-dinitrophenol (DNP) was only 1 per cent of that without DNP. Puromycin reduced leucine incorporation into uredospore protein 30 per cent, but was without effect on *A. niger* conidia.

THE EFFECT OF PUROMYCIN ON FUNGUS SPORES

Acetate. The data of Table VI show the effect of puromycin on some fungus spores. Total consumption of acetate by uredospores was enhanced, while that by conidia was not. The most significant change by uredospores was a marked increase in accumulation of amino acids. Both the per cent distribution of label in the protein fraction and its specific activity were increased as a result of the treatment. Exposure of uredospores to puromycin increased the protein nitrogen 120 per cent in *U. phaseoli* and 250 per cent in *P. sorghi* uredospores. Other experiments have shown that treatment with puromycin also resulted in a 40 per cent increase in CO_2 production in these spores relative to that retained as substrate products. Puromycin did not alter the degree of germination of the spores, but the germ tubes of the treated spores were about one-fifth the length of germ tubes from untreated spores.

The effect of varying concentrations of puromycin on specific activity of amino acids in uredospores of *U. phaseoli* is shown in Figure 6. The radioactivity of the amino acid fraction increased logarithmically with increasing amounts of puromycin. Concentrations as high as 200 p.p.m. continued to yield increasing amounts of amino acids. The specific activity of the protein fraction and protein N increased with increasing levels of puromycin up to 20 p.p.m. (Fig. 7) and then remained approximately constant.

TABLE VI

COMPARATIVE EFFECT OF PUROMYCIN ON C^{14}-ACETATE UTILIZATION BY FUNGUS SPORES

Fungus species	Puromycin, p.p.m.	Protein nitrogen, mg.	C^{14}-assimilated, c.p.m. $\times 10^{-4}$ per mg. protein N	Distribution of C^{14} in various fractions, %					
				Amino acids	Organic acids	Sugars	Nucleic acids	Crude proteins	Glucosamine
U. phaseoli	0	0.14	32	21	10	40	15	8	6
	80	0.28	53	60	5	9	6	12	7
P. sorghi	0	0.42	60	13	35	32	9	5	6
	80	0.66	119	25	15	26	20	9	4
A. niger	0	0.60	380	9	5	7	13	64	Trace
	80	0.60	370	9	5	7	13	63	Trace

Spores were germinated 6 hours with $0.005M$ glucose and 5 μc. sodium acetate-2-C^{14}. Orange juice (2% v/v) was employed with conidia of *A. niger*. Germination in all cases was 90 to 93 per cent.

The effect of 80 p.p.m. of puromycin on the specific activity of both free- and protein-bound amino acids is given in Table VII. All the amino acids except the aromatic amino acid showed an increase in specific activity.

Glucose and glutamate. The effect of puromycin on the consumption of various substrates excluding acetate by *P. sorghi* uredospores is shown in Table VIII. Puromycin appears to decrease the consumption of glucose, but the distribution of C^{14} was not appreciably changed from the control. On the other hand, puromycin caused a 2.5-fold increase in the consumption of glutamate, but only one-half as much C^{14} was incorporated into protein.

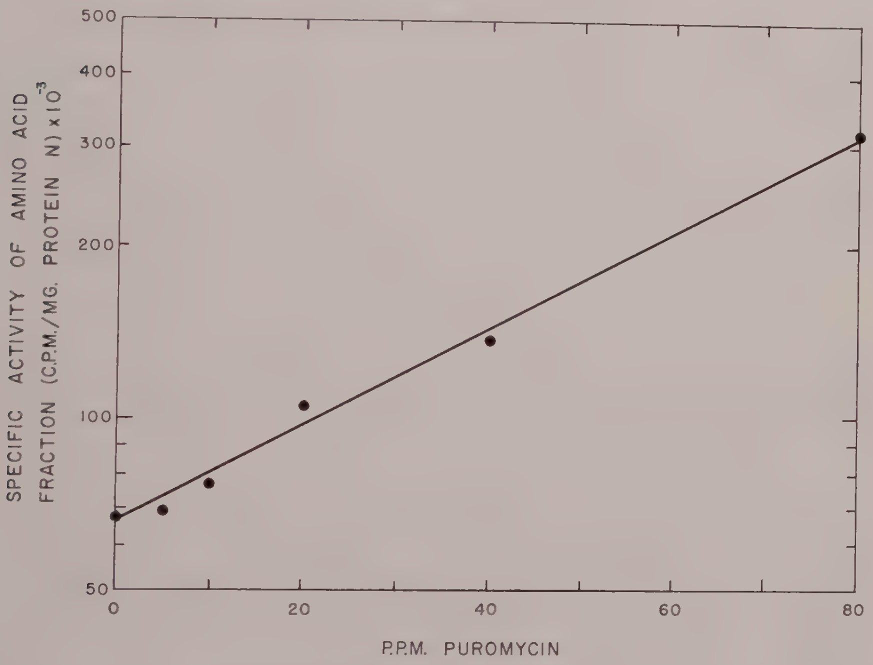

FIGURE 6. The effect of puromycin on incorporation of C^{14} from acetate-2-C^{14} into amino acids of *U. phaseoli* uredospores after incubation in the medium as in Table VI for 6 hours.

Leucine. The effect of puromycin on leucine incorporation by *P. sorghi* uredospores was explored because leucine is a convenient metabolite to study protein turnover (14), and puromycin is an inhibitor of protein synthesis (37). Leucine incorporation was measured with substrates of acetate, glucose, and several amino acids. Eighty p.p.m. of puromycin inhibited leucine incorporation into the protein to the extent of ap-

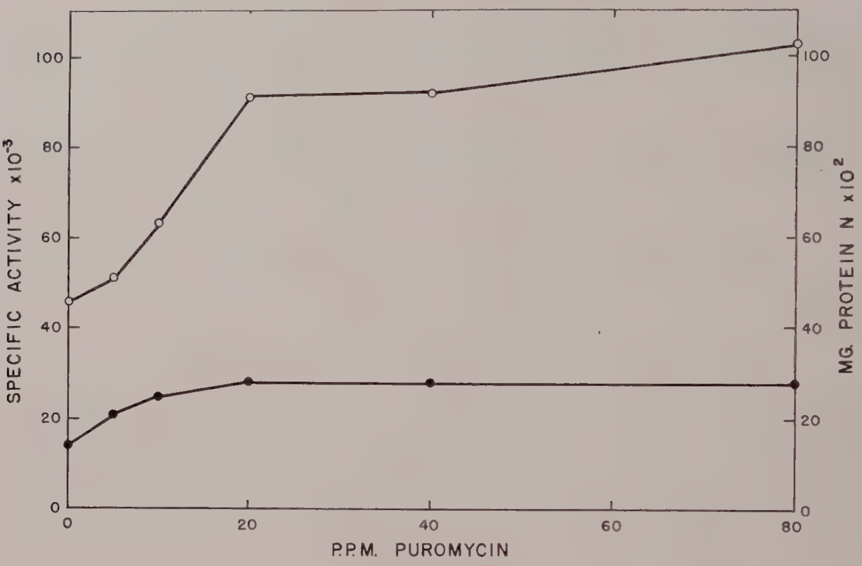

FIGURE 7. The incorporation of C^{14} from acetate-2-C^{14} into proteins of *U. phaseoli* uredospores when germinated for 6 hours on the medium as in Table VI, supplemented with puromycin. Open circles = specific activity; solid circles = protein N.

TABLE VII

SPECIFIC ACTIVITIES OF AMINO ACIDS FROM PUROMYCIN-TREATED UREDOSPORES OF P. SORGHI

Amino acid	Free amino acids		Protein amino acids	
	Control	Puromycin	Control	Puromycin
	C.p.m. per micromole			
Alanine	1,780	10,300	212	437
Arginine	—	—	67	153
Asparagine	—	10,000	—	—
Aspartic acid	3,200	14,700	852	2,861
Glutamic acid	6,952	12,355	284	1,193
Glutamine	4,000	8,000	—	—
Glycine	1,273	2,214	56	259
Leucine(s)	340	3,700	41	116
Lysine	875	—	41	181
Methionine	—	—	125	406
Phenylalanine	—	—	0	0
Proline	—	—	150	627
Serine	2,000	3,200	571	788
Threonine	2,400	5,500	62	228
Tyrosine	—	—	0	0
Valine	900	5,500	62	135

Spores germinated 6 hours with 5 μc. sodium acetate-2-C^{14}, 0.01M KH_2PO_4, and 0.005M glucose. Eighty p.p.m. puromycin was used. Methionine was determined as the sulfoxide.

TABLE VIII

EFFECT OF PUROMYCIN ON SUBSTRATE ASSIMILATION BY UREDOSPORES OF P. SORGHI

Substrate	Puromycin, p.p.m.	Protein nitrogen, mg.	C^{14} assimilated, c.p.m. $\times 10^{-4}$ per mg. protein N	Distribution of C^{14} in various fractions, %					
				Amino acids	Organic acids	Sugars	Nucleic acids	Crude proteins	Glucosamine
Glucose-U-C^{14}	0	0.5	39	3	46	—	30	11	9
	80	0.5	20	5	40	—	36	11	8
L-Glutamate-1-C^{14}	0	1.5	37	—	76	6	6	9	1
	80	1.4	93	—	88	7	2	2	Trace
L-Leucine-U-C^{14}	0	0.49	10	—	12	11	21	53	3
	80	0.45	10	—	18	16	22	36	7

Spores were germinated 6 hours with 0.005*M* glucose, 0.01*M* KH_2PO_4, and 5 μc. radioisotope. Germination was 97 to 99 per cent in all cases.

proximately 30 per cent. Thus in spite of the fact that puromycin produced a net conversion of acetate into protein (Table VI), puromycin appeared to be an inhibitor of protein synthesis when leucine was employed.

DISCUSSION

The present studies indicate that uredospores can synthesize amino acids and are able to incorporate them into protein. Although many studies have shown that uredospores are capable of a wide range of synthetic reactions (3, 5, 7, 11, 22, 23, 28, 30, 31, 33), their inability to effect a net synthesis of proteins and nucleic acids could be the crux of the obligate parasite problem. The situation seems quite analogous to that for the saprophytic conidia in which a net protein or nucleic acid synthesis did not occur until suitable nutrients were included in the medium, even though they could readily synthesize the required intermediates (12). Failure of net protein synthesis can occur before one amino acid becomes limiting (4, 13). If uredospores eventually prove to have the usual spectrum of synthetic capacity, as seems indicated, their failure to grow may result from a lack of a required nutrient or they may synthesize an inhibitor which must be removed by the host tissue.

The data in this and the earlier papers (28, 29) suggest that the rate of synthesis of certain intermediates by uredospores may be suppressed. Saprophytic fungi showed an initial burst of synthetic activity (28) even when prevented from growing by the omission of an essential nutrient. No such initial burst was observed in uredospores, even during germination (Figs. 3 and 4). In this regard puromycin had quite interesting effects (Table VI). It induced a strong increase in amino acid synthesis, while amino acid incorporation into protein was inhibited (Table VIII). Thus, when protein turnover was inhibited, amino acids accumulated and total assimilated products increased. Where such an accumulation did not occur, as with glucose, total assimilation did not increase. If an internal suppression of metabolism does exist, it might merely be a part of the syndrome resulting from the need for an essential nutrient, or it might result from an internal inhibitor which must be removed by the host.

Conidial germination is accompanied by protein and nucleic acid synthesis (28, 36). Metabolic intermediates are rapidly made from the substrate and they accumulate in the 80 per cent ethanol extract inversely to the rate of protein synthesis (Figs. 3 and 4). Uredospore germination does not result in a net synthesis of proteins and nucleic acids, but intermediates gradually accumulate. Uredospores consumed a smaller portion of the substrates tested and attained equilibrium earlier than did the conidia (Table II and Fig. 3). Conidia of *A. niger* synthesized approximately 3 times as much of the metabolic intermediates as did *P. sorghi* uredospores; nevertheless, 71 per cent of the total label consumed by uredospores remained in the 80 per cent ethanol fraction compared to 42 per cent in *A. niger* conidia (Table II). The low substrate consumption by uredospores appears to be the result of their failure to synthesize proteins and nucleic acids from the components of the metabolic pools. These intermediates then accumulated until equilibrium was reached. The slight effectiveness of most inhibitors against uredospore metabolism affords verification of this concept.

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AUTOMATIC ANALYSIS OF SUGARS SEPARATED BY COLUMN CHROMATOGRAPHY[1]

RIKSH SYAMANANDA,[2] RICHARD C. STAPLES, AND RICHARD J. BLOCK[3]

SUMMARY

A completely automated system for the determination of sugars is described. Sugars were chromatographed on a Dowex 1, borate-form, chromatographic column using a sodium chloride-borate gradient controlled by a Varigrad. A portion of the effluent from the column was monitored continuously using either anthrone or cysteine in sulfuric acid. The reagent and effluent were mixed, heated to 95° C., and the color measured and recorded automatically. Approximately 4 μg. of each sugar was required for analysis. The recovery was better than 95 per cent using 0.25 to 1 mg. of each sugar. Reproducibility was in the order of ±1 per cent.

INTRODUCTION

The quantitative analysis of sugars by paper chromatography is not satisfactory when only small amounts of sugars are available. The more sensitive procedure of Khym and Zill (5) utilizing chromatography on a strongly basic anion exchanger is tedious. This paper describes a method for determining automatically a wide variety of sugars in micro-quantities with considerable accuracy.

MATERIALS AND METHODS

Six hundred grams of strongly basic anion exchange resin, Dowex 1-X8, chloride-form (200 to 400 mesh), was suspended in 1 liter of deionized water. After allowing 15 minutes for settling, colloidal material and fine particles were removed by decantation. After the fines had been removed, the resin was converted to the borate form by passing a 0.25 M solution of potassium tetraborate through the resin bed until the effluent showed a negative test for chloride ion with silver nitrate. The resin was washed with water to remove excess borate and resuspended in 0.001 M solution of $K_2B_4O_7 \cdot 4H_2O$. Resolution of the sugars was not improved by hydrolytic grading of the resin (4). The resin was packed under pressure in a 0.9×100 cm. column. After the column had been washed with about 50 ml. of 0.001 M $K_2B_4O_7 \cdot 4H_2O$, a mixture of sugars dissolved in 10 ml. of the same solution was introduced into the column.

The sugars were resolved by the borate-chloride exchange method using the variable gradient device (Varigrad) of Peterson and Sober (8).

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[3] Deceased February 4, 1962.

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Chambers 1, 3, 5, and 6 contained 0.05 M $Na_2B_4O_7 \cdot 10H_2O$ adjusted to pH 8.0. Chambers 2, 4, and 7 contained NaCl in addition so that the initial concentration of NaCl was 0.001 M, 0.055 M, and 0.300 M, respectively. Each chamber contained 230 ml.

The effluent stream was divided by using an h-shaped glass tube so that one-half entered the analytical system and the other half an automatic fraction collector (Fig. 1). The outflow rate from the column was regulated by means of a proportioning pump.[4] The manifold assembly (Figs. 1 and 2) consisted of Tygon[4] and Acidflex 5000[4] with specified lumens which allowed 1 part of sample to be added to 4 parts of reagent. The sample and air tubes were connected to an h-shaped glass tube so that air and sample flowed toward the reagent supply together (Fig. 3). By using a short piece of Tygon sleeving the "h" fitting was attached to the built-in "T" (Fig. 3) of the glass mixing coil, where the sample was layered on top of the reagent, and simultaneously was segmented by the air flowing in with the sample.

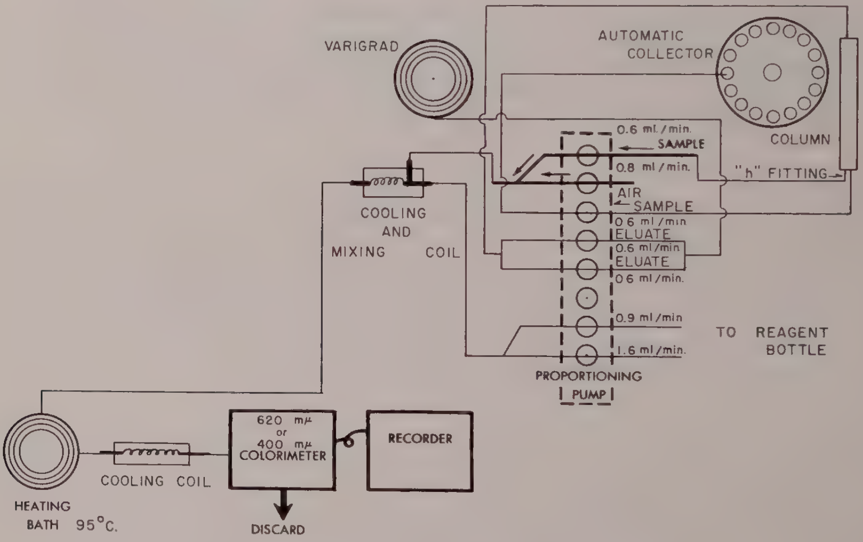

FIGURE 1. Flow diagram for automatic sugar analyses.

The anthrone (6) or cysteine (3) methods were employed for quantitative analysis of sugars in the effluent stream. Reagents were prepared daily. The anthrone reagent contained 1 g. of anthrone in 1 l. of 98 per cent sulfuric acid. The cysteine reagent contained 750 mg. cysteine hydrochloride in 1 l. of 84 per cent sulfuric acid.

The reaction mixture was cooled in a water-jacketed mixing coil to prevent premature color development. The color reaction was developed

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[4] Technicon Chromatography Corp., Chauncey, N. Y.

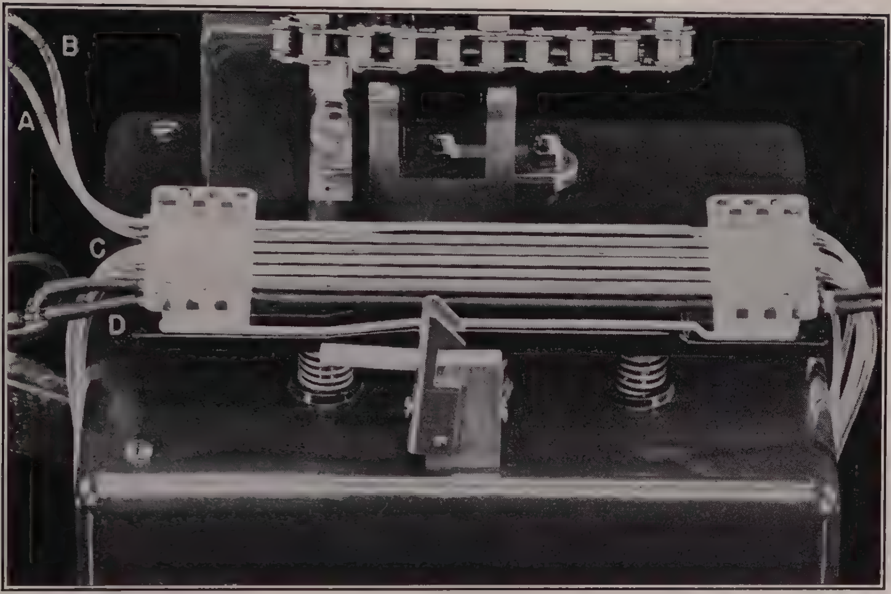


FIGURE 2. View of pump showing manifold assembly. Hosing of manifold consists of (A) sample line for anthrone reaction, (B) air line, (C) sample line to supply fraction collector, and (D) reagent lines.

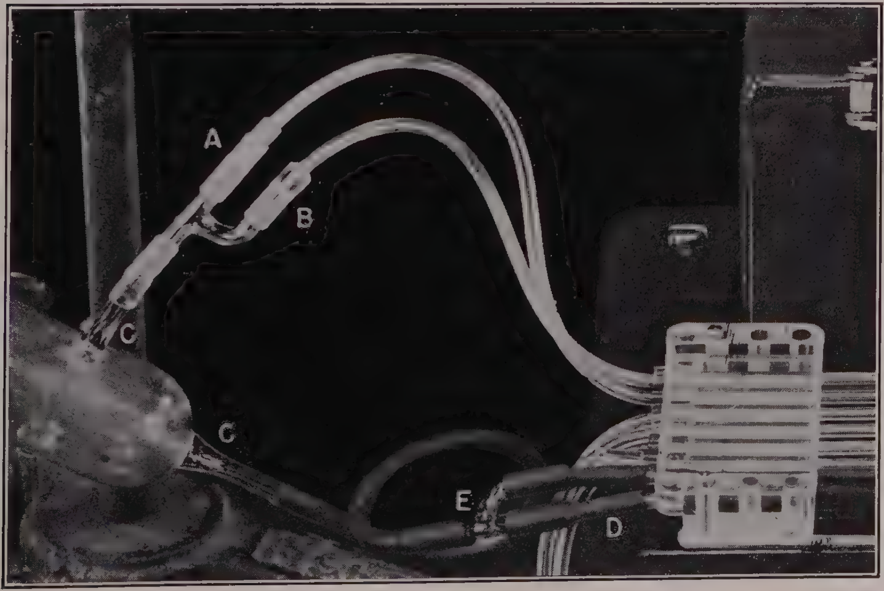


FIGURE 3. View of h-shaped glass tube showing arrangement of (A) air line, (B) sample line attached to built-in T-fitting, (C) cooling-mixing coil, (D) anthrone supply lines which merge at (E) h-shaped glass tube connected to T of the mixing coil.

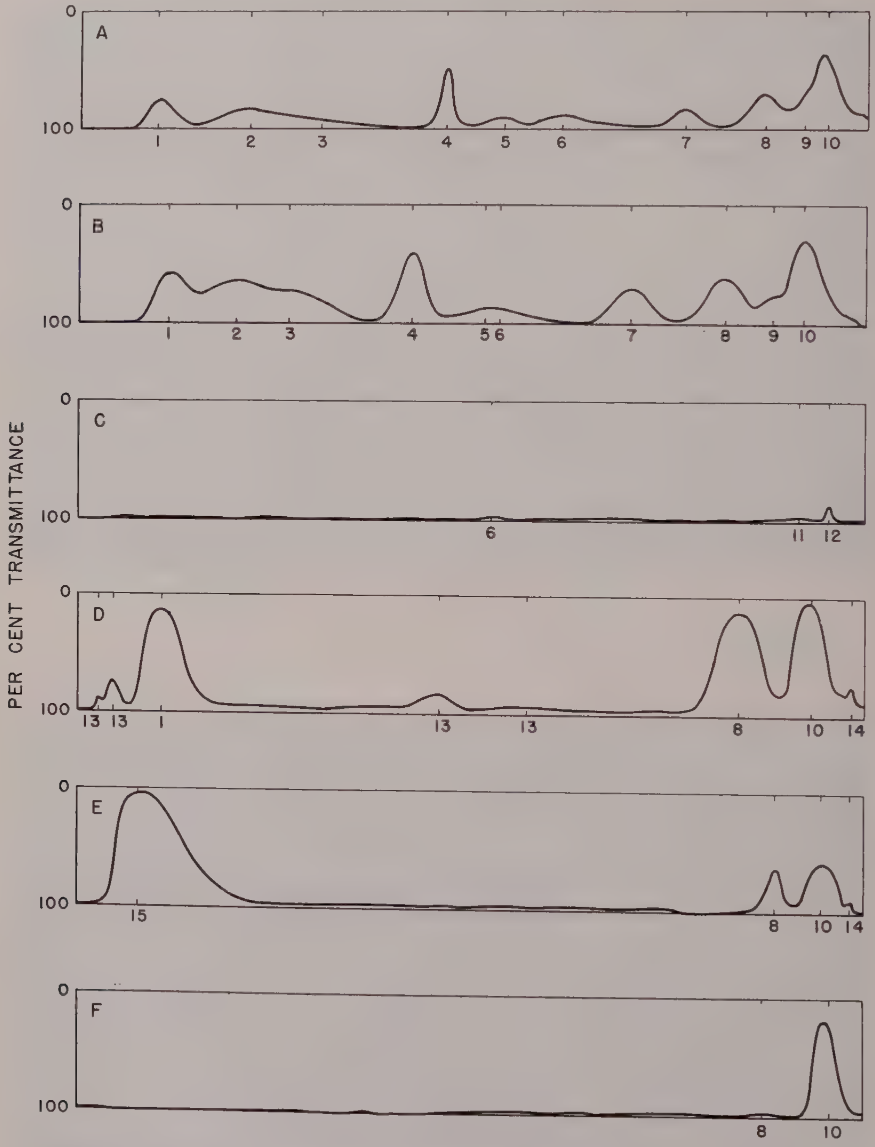


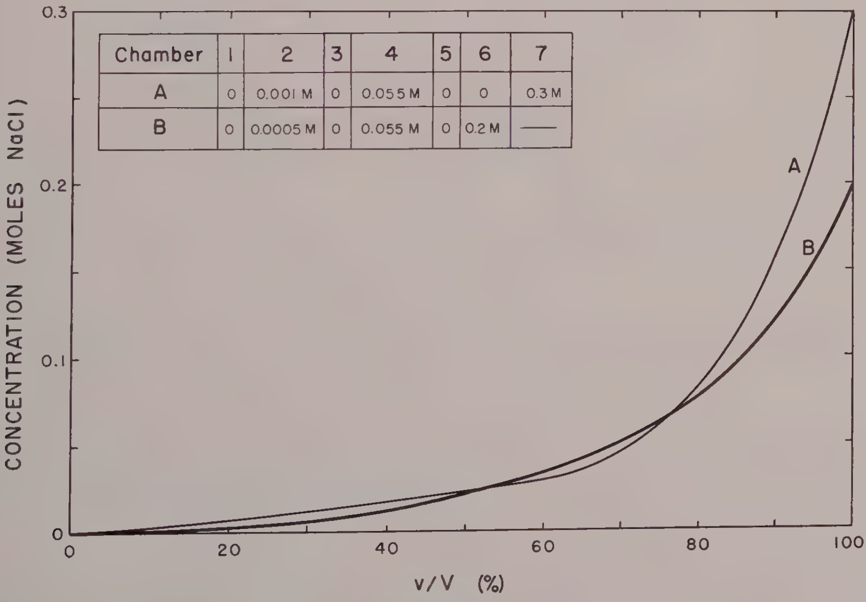
FIGURE 4. Separation of sugars by ion exchange chromatography. Hexose standards were detected with (A) anthrone, (B) cysteine, while (C) pentoses were detected with cysteine. The cysteine method was used to assay sugars from (D) rust-infected corn leaves, (E) corn rust uredospores, and (F) human blood. Sugars employed or detected were (1) sucrose, (2) raffinose, (3) cellobiose, (4) maltose, (5) melibiose, (6) ribose, (7) mannose, (8) fructose, (9) galactose, (10) glucose, (11) arabinose, (12) xylose, (13) unknown, (14) artifact, and (15) trehalose.

and reached a maximum after 15 minutes of 95° C. After emerging from the heating bath, the stream was cooled using another water-jacketed coil before entering the colorimeter through the flow cuvette. The color was recorded automatically at 620 mμ (with anthrone), or 400 mμ (with cysteine). The area under each peak was used to estimate the quantity of sugars.

After chromatography, tubes from the fraction collector containing the sugars were pooled, the borate removed by distillation *in vacuo* with absolute methanol (9), and each sugar was identified by paper chromatography (1, 2). Radioactivity in the fractions can also be determined using conventional counting techniques.

RESULTS AND DISCUSSION

The separation of sucrose, raffinose, cellobiose, maltose, ribose, melibiose, mannose, fructose, galactose, glucose, arabinose, xylose, and trehalose is shown in Figure 4 A and B. The borate-sodium chloride gradient shown in Figure 5 A was used. The resolution of sucrose, raffinose, and cellobiose was improved using the elution gradient shown in Figure 5 B.



Chamber	1	2	3	4	5	6	7
A	0	0.001 M	0	0.055 M	0	0	0.3 M
B	0	0.0005 M	0	0.055 M	0	0.2 M	——

FIGURE 5. Sodium chloride-borate gradients. (A) Gradient employed for best separation of most sugars, and (B) gradient employed for best separation of sucrose, raffinose, and cellobiose. Inset table depicts arrangement of Varigrad. All chambers contain 0.05 *M* $Na_2B_4O_7 \cdot 10H_2O$, at molarities indicated. v/V (%) indicates output volume as % of total volume.

For this gradient, six chambers of the Varigrad were filled as shown in Figure 5 B. However, the resolution of other sugars was better and the peaks were sharper in chromatograms obtained with the former gradient (Fig. 5 A).

Sharper peaks resulted when the cysteine method of color development was employed (Fig. 4 B). Figure 6 shows absorption spectra of the ribose, glucose, and raffinose chromogens. The most suitable wavelength for analysis of a mixture of hexoses and pentoses lies in the region between 395 and 400 mμ. However, a 410 mμ filter has been used in these experiments because a filter in the range 395 to 400 mμ was not available. The 410 mμ filter resulted in an inadequate optical resolution of melibiose and ribose (Fig. 4 B).

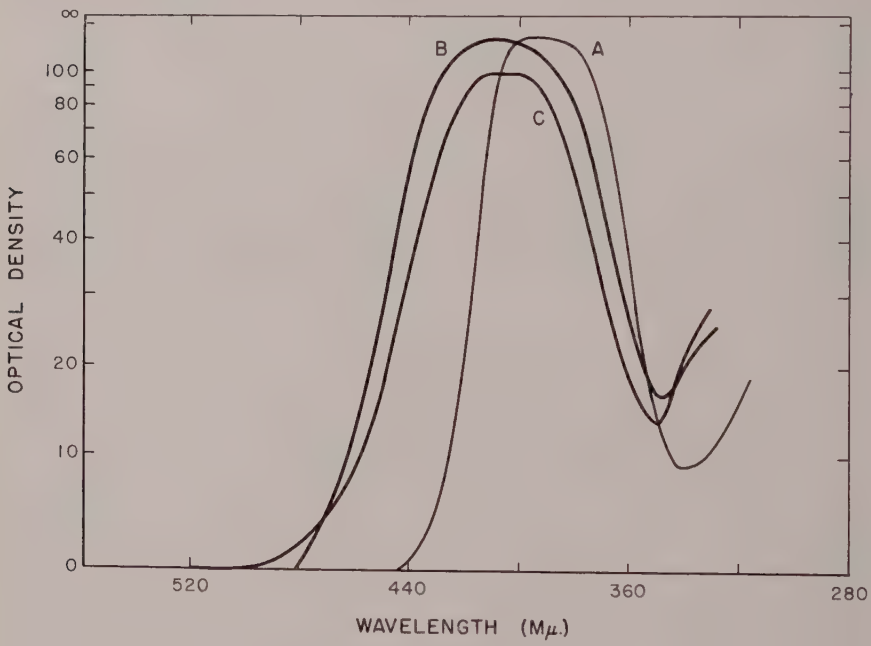


FIGURE 6. Absorption spectra of cysteine chromogens. (A) Ribose, (B) glucose, and (C) raffinose.

Extracts of infected corn leaves, corn rust uredospores, and blood serum were chromatographed on borate columns by development with the gradient of Figure 5 A. Analysis of the effluents is shown in Figure 4 D, E, and F. Free sugars and cysteine-positive materials in the leaf extracts were eluted from the columns in the following order: U_1, U_2, sucrose, U_3, U_4, fructose, and glucose (Fig. 4 D). Sugars in corn rust uredospores consisted of trehalose, fructose, and glucose (Fig. 4 E). A large quantity of glucose and a trace of fructose were found in the sample of blood serum (Fig. 4 F). If only sucrose, glucose, and fructose are present in a mixture, a 10 cm. column and the linear gradient of sodium chloride described by Parr (7) will provide adequate resolution. Six hundred and fifty milliliters of 0.2 *M* sodium chloride in the reservoir chamber was best for this purpose.

Inspection of Figure 4 B and C shows that ribose was completely resolved whereas arabinose and xylose were eluted with fructose and galactose, respectively. Even though complete separation of pentoses and hexoses was not possible under these conditions, a quantitative estimation could be made by determining elution characteristics and peak-to-volume values for synthetic mixtures of hexoses and pentoses. Differences in peak-to-volume values would represent the amount of hexoses in the mixture.

The area under each curve was proportional to the quantity of sugar added to the column. Recoveries averaged better than 95 per cent over the range of 0.25 to 1 mg. Reproducibility was in the order of ± 1 per cent. The threshold for detection of the sugars was about 4 micrograms.

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BIOCHEMICAL AND PHYSIOLOGICAL STUDIES OF TISSUE CULTURES AND THE PLANT PARTS FROM WHICH THEY ARE DERIVED. III. PAUL'S SCARLET ROSE

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AND HENRY J. LAURENCOT, JR.

SUMMARY

A time-course study of changes in a stem tissue culture from Paul's Scarlet rose (*Rosa* sp.) was carried out, and was compared with mature stems and leaves of the parent plant. The tissue contents of free and bound amino acids, nonvolatile organic acids, sugars, and ribo- and deoxyribonucleic acids were compared. Results are presented for tissue cultures on the basis of amount of constituents per culture and per gram of dry weight, and for stems and leaves on the basis of per gram of dry weight.

Results showed that nuclear divisions in the tissue cultures cease after three weeks. This is generally correlated with decreasing or leveling off of total free and bound amino acids, sugars, and nucleic acids per culture. Changes in individual constituents are presented.

INTRODUCTION

This is the third article in a series of studies comparing the biochemistry of plant tissue cultures with the biochemistry of the plant parts from which they were derived. The first article in this series was concerned with *Agave toumeyana* Trel. (20) and the second with *Ginkgo biloba* L.(17). Outstanding differences between the leaves of *Agave* and a tissue culture derived from the leaves were found in the content of free and combined hydroxyproline, the ratios of malate to citrate, of ribonucleic to deoxyribonucleic acid, and the contents of glucose, fructose, and sucrose. The *Ginkgo* tissue culture from pollen was compared with several tissues of the intact plant, including germinated and nongerminated pollen. The tissue culture resembled germinated pollen more than it did the other tissues analyzed.

The present paper is concerned with changes occurring in a tissue culture from the young stems of the Paul's Scarlet rose (*Rosa* sp.) with the stems and leaves themselves. Interest in this particular plant and the tissue culture from it arose from two separate lines of investigations being carried out by the authors. The first was a study of the physiology and biochemistry of roses (18, 19, 21, 22, 23), the second was a study of the submerged growth of plant tissues and cells (7, 14, 15). The tissue culture from the stem of Paul's Scarlet rose grows extremely fast and, as a result, many growth studies have been carried out with this tissue. The facts that

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it (a) will grow on a basal medium supplemented with either coconut milk or a mixture of yeast and malt extracts (14), (b) has different growth characteristics on each of these media, (c) has a wide temperature tolerance (15), and (d) can be produced in large amounts (7, 14, 15), make an analysis of its constituents and a knowledge of its growth requirements all the more important.

It should be emphasized that the stems used in the present investigation and the stems used for the establishment of the tissue culture were from the same plant.

MATERIALS AND METHODS

Establishment of tissue culture. The tissue culture used in the present work was isolated in October, 1957 (6). Sections of young stem material were surface sterilized with mercuric chloride solution (1:2000) containing a trace of OT aerosol and placed on White's basal medium (24, p. 103) supplemented by 2,4-D (6 p.p.m.) and coconut milk (18 per cent by volume) (10). This medium is designated as W-2,4-D-CM. The coconut milk was prepared as previously described (20). After the appearance of callus, the tissue was cut off from the original stem and subcultured on the W-2,4-D-CM medium.

The growth cycle of the stem tissue culture is typical for an organism growing in an essentially closed growth system, i.e., there is a lag phase, a period of rapid proliferation and then decline. The system is characterized as closed because no provision is made for additions to the medium; hence, one or more nutrients become limiting with time. On a solid agar medium the tissue grew rapidly (Fig. 1), but this rate of growth was not maximal. Previous studies using a carboy culture apparatus gave much better growth when liquid media, aeration, and agitation were employed (14, 15).

Experimental culture techniques. Experimental culture procedures and growth conditions were the same as used previously (6, 20). The cultures used for analyses were grown on W-2,4-D-CM agar medium, on which the stem tissue cultures formed a homogeneous mass of cells, white to pale yellow in color and loose in texture. Microscopic examination showed that the tissue was composed of vacuolate parenchyma and meristematic cells. The cells were spherical and relatively uniform in size, and contained nuclei which were observed to vary in chromosome number. No differentiation was observed.

Cytological examinations of Paul's Scarlet rose stem tissue were made at two-day intervals for four weeks in order to count the frequency of nuclear divisions. The results from these observations (Fig. 2) indicated that many nuclear divisions were taking place from the second day after

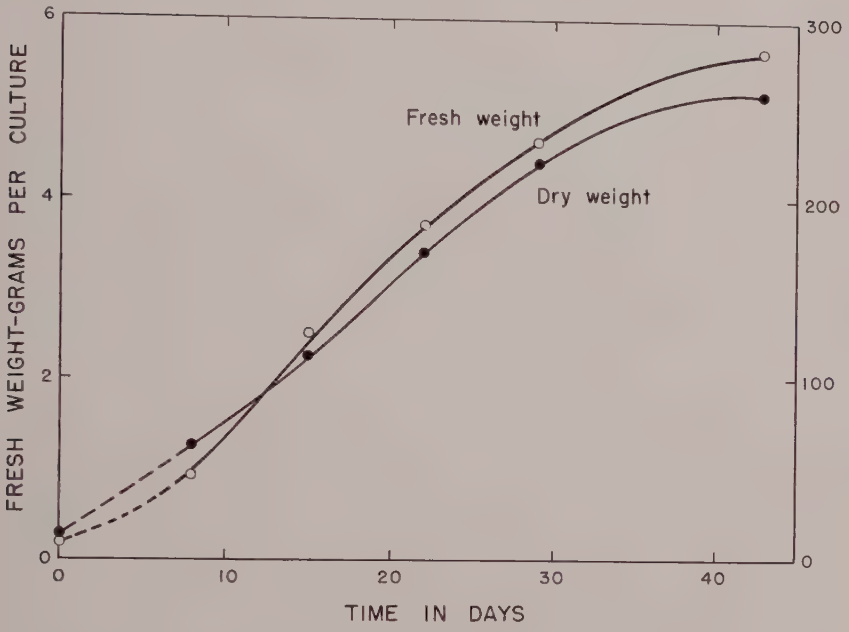

FIGURE 1. Fresh and dry weights of Paul's Scarlet rose stem tissue cultures at five stages of growth.

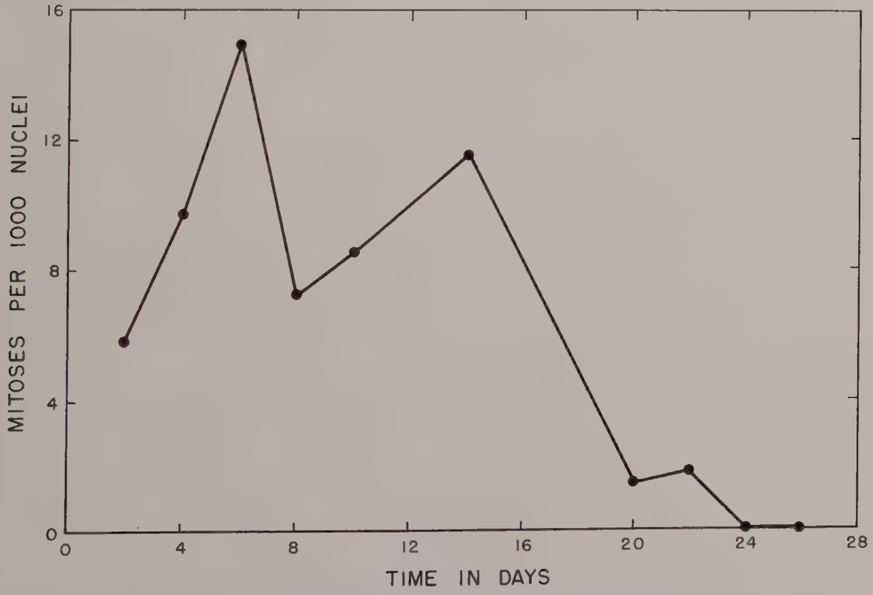

FIGURE 2. Frequency of nuclear divisions in Paul's Scarlet rose stem tissue cultures at different stages of growth.

transfer through the second week. Thereafter the frequency declined and no division figures were observed after the third week.

Analytical techniques. The methods of preparing tissues for analysis, and the quantitative determinations of free amino acids, bound amino acids, nonvolatile organic acids, and sugars have been previously reported (20). Proline was determined by the method of Thompson and Morris (13) and hydroxyproline by the method of Leach (5). Ribonucleic acid (RNA) nucleotides were liberated from the alcohol-insoluble residue and determined as described by Porter and Weinstein.[2] Deoxyribonucleic acid (DNA) was extracted by the method of Smillie and Krotkov (9) and determined by the deoxyribose method of Burton (1).

RESULTS

Changes in composition from the time of transfer of inoculum, which was 21 days old, to the first harvest after 8 more days, are not shown in the subsequent figures and tables. However, it is important that the comparison be taken into consideration. Since cultural conditions were kept as uniform as possible, and since the tissues were normally transferred after 21 days, one may assume that the composition of the inoculum was similar to that reported for the 22-day harvest.

Results of fresh and dry weight determinations are illustrated in Figure 1.

When the free amino acid data were expressed on a per culture basis (Fig. 3), alanine and γ-aminobutyric acid reached their maximum concentration at 8 days, after which they declined. Glutamic acid, valine, and leucines were highest at 15 days; aspartic acid, proline, hydroxyproline, and phenylalanine at 22 days; and serine, glycine, threonine, arginine, β-alanine, glutamine, and methionine sulfoxide at 29 days. By 43 days, all amino acids were lower. The patterns for lysine, tyrosine, homoserine, and asparagine were variable, although each of them fell off by the 43-day harvest. Total free amino compounds rose from 8 days to a relatively constant level at 15, 22, and 29 days, but by 43 days it had dropped back to the 8-day level. The same data expressed on a dry weight basis are shown in Table I. Alanine, valine, leucines, phenylalanine, tyrosine, γ-aminobutyric acid, homoserine, β-alanine, glutamine, and asparagine were all at their maximum concentration at 8 days. Aspartic and glutamic acids, serine, glycine, threonine, lysine, and arginine were highest at 15 days, while proline and hydroxyproline reached their maxima at 22 days. Regardless of the means of expression, aspartic and γ-aminobutyric acids were highest at 8 days, glutamic acid at 15 days, and proline and hydroxyproline at 22 days.

It is of interest to note (Table I) that the dominant free amino compound of stem tissue cultures changed at different ages. Thus, alanine was

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[2] PORTER, CLARK A., and LEONARD H. WEINSTEIN. Unpublished data.

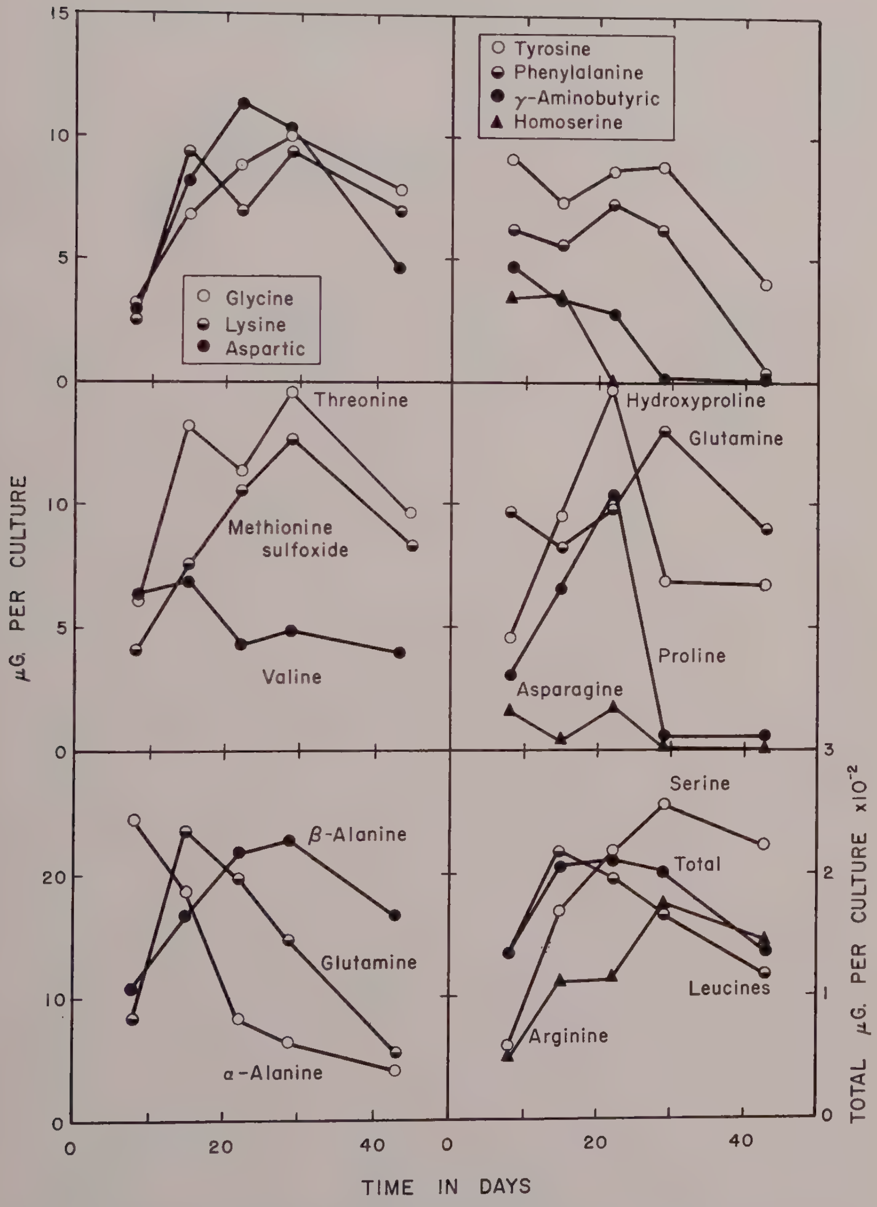

FIGURE 3. Free amino acid and amide content of Paul's Scarlet rose stem tissue cultures at five stages of growth.

by far the major constituent at 8 days. By 15 days, the major compounds were glutamate, serine, alanine, leucines, and β-alanine; by 22 days, glutamate, serine, leucines, and β-alanine; and by 29 and 43 days, respectively, serine and β-alanine. By contrast (Table I), stem tissues contained nearly 0.4 per cent asparagine on a dry weight basis, and considerable quantities of glycine, alanine, arginine, γ-aminobutyrate, and glutamine. In leaf tissues, the major free amino compounds were glutamate, glutamine, γ-aminobutyrate, and proline. In no instance did the concentration of asparagine approach that of glutamine in stem tissue cultures in spite of its enormous accumulation in stems.

TABLE I

FREE AMINO ACID AND AMIDE CONTENT OF PAUL'S SCARLET ROSE STEM TISSUE CULTURES AT FIVE STAGES OF GROWTH, AND OF LEAF AND STEM TISSUES

Amino acid or amide	Age of tissue culture in days					Leaves	Stems
	8	15	22	29	43		
	μG. per gram dry weight						
Aspartic	48.3	72.9	65.7	46.4	18.5	58.4	61.5
Glutamic	132.2	211.5	115.0	66.5	21.1	402.5	80.6
Serine	94.8	151.5	126.1	116.5	89.5	130.4	116.6
Glycine	51.3	60.4	50.0	45.6	31.0	38.3	229.6
Threonine	99.6	118.5	65.1	65.7	39.5	68.3	92.0
Alanine	402.0	169.0	47.0	28.9	15.7	72.0	258.4
Lysine	40.2	83.5	39.4	42.4	27.7	28.1	18.3
Arginine	84.8	99.5	67.3	81.5	57.0	87.0	228.0
Proline	47.5	59.2	60.2	Trace	Trace	238.4	127.1
Valine	102.5	61.6	24.5	20.6	15.6	76.2	79.2
Leucine(s)	218.0	196.0	113.8	75.9	47.5	128.6	84.8
Phenylalanine	99.5	49.0	42.0	27.9	Trace	38.8	19.9
Tyrosine	148.2	65.5	49.0	38.9	17.0	46.8	21.6
γ-Aminobutyric	76.0	30.0	16.4	Trace	Trace	163.2	252.1
Homoserine	57.0	31.1	—	—	—	—	—
Methionine sulfoxide	66.7	77.7	62.0	57.9	34.0	49.1	26.0
Hydroxyproline	71.9	85.3	87.0	31.5	27.4	—	—
Asparagine	26.7	2.9	9.2	Trace	Trace	35.1	3,626.3
Glutamine	158.3	74.8	57.7	59.3	36.4	688.2	228.5
β-Alanine	175.0	150.0	126.4	103.0	68.1	93.0	29.9

The composition of the bulk proteins of the various tissues is shown in Figure 4 (mg. protein amino acid per culture). Although most of the protein amino acids followed a definite pattern, reaching a maximum concentration at 29 days, there were certain outstanding exceptions. Lysine, arginine, and tyrosine, for example, attained their highest concentration in tissue cultures at 15 days, while phenylalanine reached its peak at 22 days. Proline and hydroxyproline were distinctly different from the other protein amino acids in that they increased throughout the growth period. The total amount of protein amino acids per culture reached a maximum at 29 days, then fell off, presumably due to proteolysis. It is

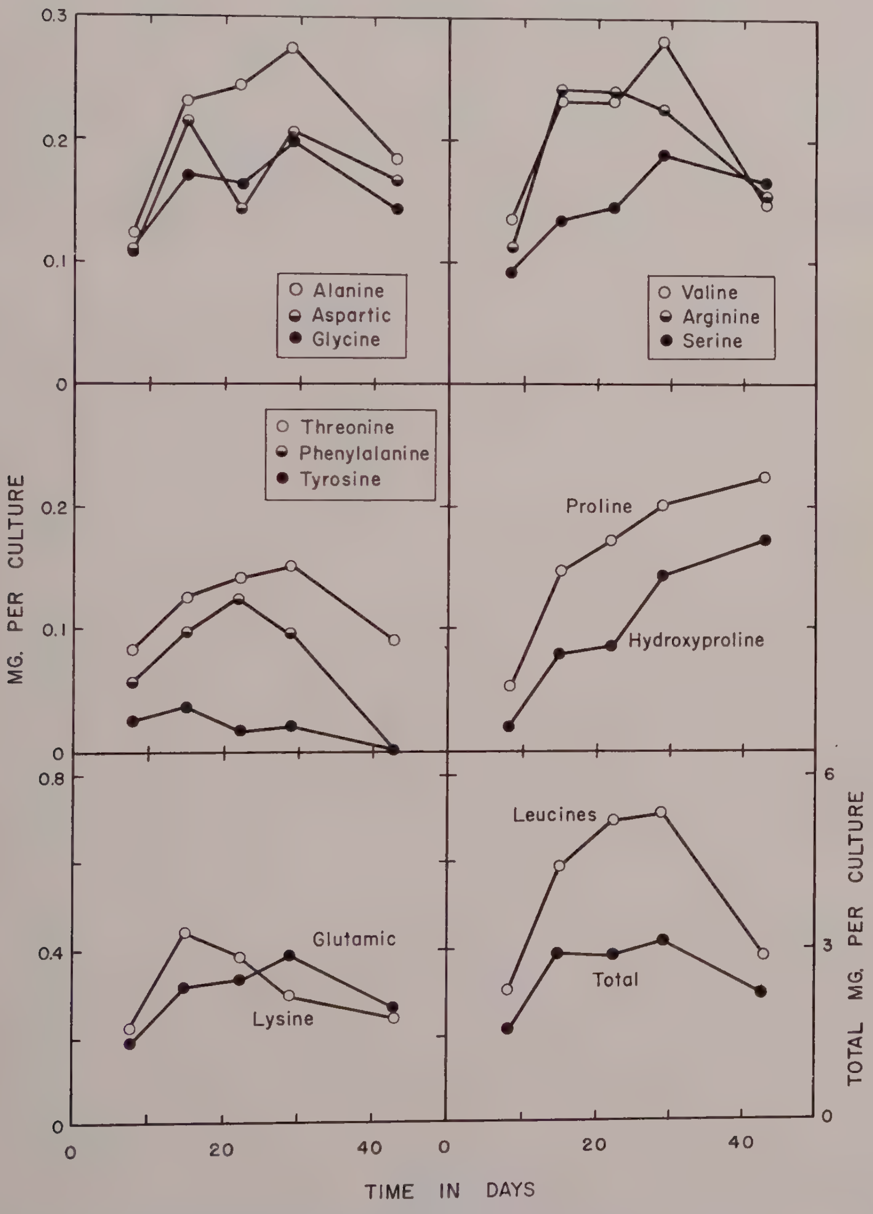

FIGURE 4. Bulk protein amino acids of Paul's Scarlet rose stem tissue cultures at five stages of growth.

interesting that ribonucleic acid was still being synthesized during the period of protein loss. When the amino acid nitrogen was expressed as per cent of protein nitrogen (Table II), a somewhat different and less distinct pattern emerged. Thus, the proteins were highest in lysine and tyrosine at 8 days, in arginine, leucines, and phenylalanine at 22 days, in glutamate at 29 days, and in serine, proline, and hydroxyproline at 43 days. Comparing results for the oldest stem tissue cultures (43 days) with stems, the salient differences were found in concentrations of proline and hydroxyproline, and in the presence of cysteic acid and methionine sulfoxide in the stems (at least at detectable levels).

TABLE II

BULK PROTEIN AMINO ACID CONTENT OF PAUL'S SCARLET ROSE STEM TISSUE CULTURES AT FIVE STAGES OF GROWTH, AND OF LEAF AND STEM TISSUES

Amino acid	Age of tissue culture in days					Leaves	Stems
	8	15	22	29	43		
	Amino acid N as % of total determined						
Cysteic	—	—	—	—	—	—	2.3
Aspartic	4.7	5.1	3.5	4.7	5.7	7.4	5.1
Glutamic	7.6	6.8	7.5	8.3	7.9	7.3	8.8
Serine	4.9	4.2	4.6	5.6	7.1	3.7	5.8
Glycine	8.2	7.2	7.1	8.3	8.5	7.6	6.8
Threonine	3.9	3.3	3.8	4.0	3.6	3.8	4.3
Alanine	7.9	8.0	9.0	9.6	9.0	6.4	8.6
Lysine	19.9	19.1	12.5	8.1	9.0	8.3	7.7
Arginine	14.8	17.4	18.1	16.1	15.6	27.4	16.8
Proline	2.7	4.0	4.9	5.4	8.8	4.4	5.7
Valine	6.7	6.2	6.6	7.5	5.7	4.2	6.1
Leucine(s)	14.8	14.0	17.2	16.5	12.8	8.4	13.8
Phenylalanine	2.0	1.9	2.5	1.8	Trace	2.4	3.0
Tyrosine	0.9	0.7	0.4	0.4	Trace	2.2	2.0
Hydroxyproline	1.0	2.1	2.3	3.7	6.3	2.4	1.9
Histidine	—	—	—	—	—	2.2	—
Methionine sulfoxide	—	—	—	—	—	1.9	1.3

The composition of leaf proteins was found to be quite different from stems, with major differences apparent in cysteic acid, arginine, leucines, and histidine (Table II).

Comparison of molar ratios of the constituent amino acids of the bulk proteins (Table III) of the tissue cultures, stems, and leaves reveals several pertinent differences. Among the tissue cultures, less lysine was found in the protein with increasing age. On the other hand, aging tissues exhibited an enriched content of proline and hydroxyproline. Comparisons of molar ratios of stem proteins with those of the stem tissue cultures suffer from the fact that there is no way to assess the relative physiological age of these tissues. In general, however, stem proteins appeared to be somewhat lower in serine, glycine, alanine, proline, hydroxyproline, valine, and leucines than

43-day-old tissue cultures, and higher in arginine, tyrosine, and phenylalanine. The presence of hydroxyproline in leaves and stems of the rose, as well as in petals (19), is of some interest since it has not usually been found as a common constituent of mature plant tissues (11), but rather in randomly-proliferating tissues (12). However, recent studies have shown it to be associated with cell wall fractions (2, 4) of mature tissues.

Among the nonvolatile organic acids in the tissue cultures (μeq. per culture), shikimate reached its highest level at 43 days (Fig. 5), quinate at 29 to 43 days, succinate between 22 and 43 days, fumarate and α-ketoglutarate at 22 days, and malate and citrate-malonate at 29 days. Total organic acidity was highest at 29 days. When the data were expressed on a dry weight basis (Table IV), fumarate reached greatest concentration at 8 days; shikimate, quinate, malate, and α-ketoglutarate at 15 days; and glycerate, succinate, and citrate-malonate at 22 days. Total acidity was highest at 15 days. The dominant organic acid of both leaf and stem tissues was quinic acid.

TABLE III

MOLAR RATIOS OF BULK PROTEIN AMINO ACIDS OF PAUL'S SCARLET ROSE STEM TISSUE CULTURES AT FIVE STAGES OF GROWTH, AND OF LEAF AND STEM TISSUES. VALUES EXPRESSED AS MOLAR RATIO BASED ON THREONINE AS 1.00

Amino acid	Age of tissue culture in days					Leaves	Stems
	8	15	22	29	43		
Aspartic	1.33	1.80	1.09	1.42	1.85	1.56	2.11
Glutamic	1.99	2.15	2.08	2.28	2.35	1.54	1.87
Serine	1.29	1.25	1.20	1.43	1.95	0.78	1.35
Glycine	2.14	2.17	1.84	2.11	2.38	1.68	1.38
Threonine	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Alanine	2.06	2.42	2.34	2.44	2.55	1.34	1.73
Lysine	2.61	2.81	1.65	1.02	1.27	0.88	1.20
Arginine	0.97	1.31	1.19	1.02	1.09	1.46	2.00
Proline	0.70	1.21	1.28	1.39	2.40	0.92	1.43
Valine	1.76	1.88	1.70	1.97	1.58	0.90	1.11
Leucine(s)	3.89	4.24	4.50	4.07	3.61	1.79	1.97
Phenylalanine	0.53	0.56	0.63	0.46	Trace	0.51	0.49
Tyrosine	0.22	0.19	0.09	0.09	Trace	0.48	0.39
Hydroxyproline	0.24	0.58	0.55	0.86	1.62	0.52	0.31
Histidine	—	—	—	—	—	0.16	—
Methionine sulfoxide	—	—	—	—	—	0.41	0.43
Cysteic acid	—	—	—	—	—	—	0.37

Examination of the data shows that certain similarities exist in the organic acid balance between stem and leaf tissues, but not with cultured stem tissues. For example, the malate/citrate ratios in leaves and stems were 4.5 and 4.3, respectively. In tissue cultures, the ratios were 42.3, 35.9, 29.4, 34.7, and 77.4, respectively, with increasing maturity. The malate/quinate ratios of leaves and stems were 0.3 and 0.6, and for the

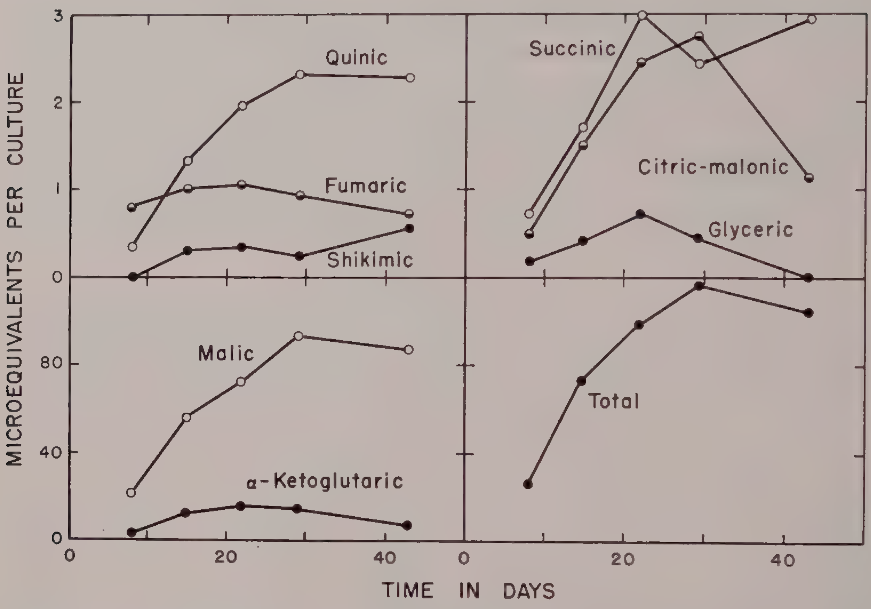

FIGURE 5. Nonvolatile organic acids of Paul's Scarlet rose stem tissue cultures at five stages of growth.

TABLE IV

NONVOLATILE ORGANIC ACIDS OF PAUL'S SCARLET ROSE STEM TISSUE CULTURES AT FIVE STAGES OF GROWTH, AND OF LEAF AND STEM TISSUES

Organic acid	Age of tissue culture in days					Leaves	Stems
	8	15	22	29	43		
	μEq. per gram dry weight						
Shikimic	0	2.78	1.96	1.05	2.27	12.6	1.9
Quinic	5.55	11.55	11.30	10.52	9.21	240.8	39.1
Glyceric+unknown	3.11	3.78	4.13	2.10	0	15.4*	1.7*
Succinic	11.48	15.12	17.63	10.93	11.93	22.0	4.7
Malic	344.00	479.00	425.00	423.00	352.00	75.7	22.7
Citric+malonic	8.15†	13.34†	14.45†	12.41†	4.55†	16.8**	5.3**
Fumaric	12.96	8.90	6.09	4.21	2.84	7.5	3.0
α-Ketoglutaric	62.30	116.30	93.80	66.80	31.55	21.0	6.9
Total	447.55	650.77	574.36	531.02	434.35	411.8	85.3

* Glyceric only.
** Citric acid only.
† Major component of fraction was citric acid.

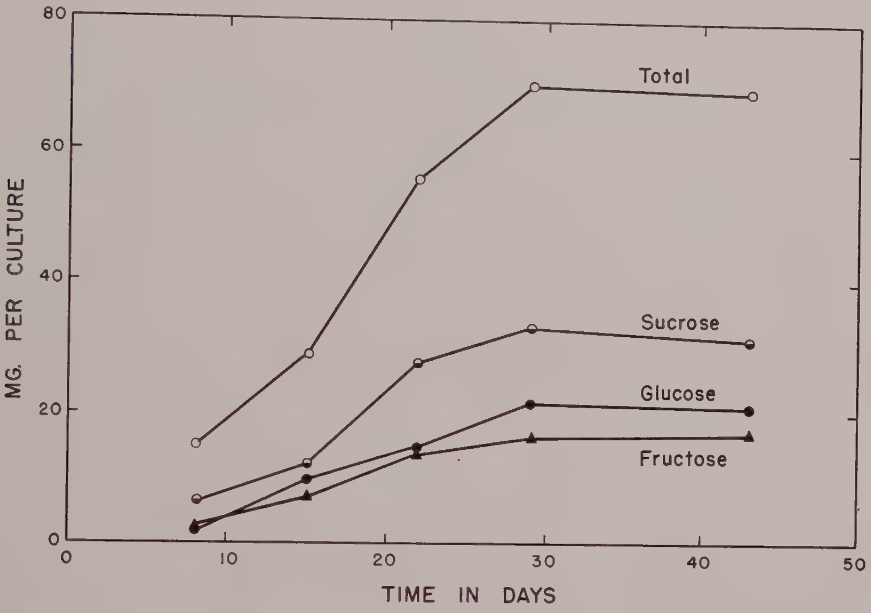

FIGURE 6. Free sugars of Paul's Scarlet rose stem tissue cultures at five stages of growth.

tissue cultures were 62.0, 41.5, 37.6, 40.2, and 37.2. Comparisons between the quinate/shikimate ratios of leaves and stems were again similar, 19.1 and 20.6, while in the tissue cultures the values were generally lower. At 8 days, no ratio could be calculated, but ratios for other ages were 4.2, 5.8, 10.0, and 4.1. Stem tissue cultures contained from 5.1 to 7.6 times as much total organic acidity as did stems, and approximately the same amount as leaves on a dry weight basis. Neither leaves nor stems contained mal-

TABLE V

FREE SUGARS OF PAUL'S SCARLET ROSE STEM TISSUE CULTURES AT FIVE STAGES OF GROWTH, AND OF LEAF AND STEM TISSUES

Sugar	Age of tissue culture in days					Leaves	Stems
	8	15	22	29	43		
	Mg. per gram dry weight						
Fructose	39.9	62.7	80.0	72.0	68.7	16.7	17.5
Glucose	35.0	83.3	84.8	94.2	85.0	18.1	16.7
Sucrose	101.8	108.0	161.2	151.3	123.3	13.0	Trace
Xylose	—	—	—	—	—	0.4	Trace
Total	176.7	254.0	326.0	317.5	277.0	48.2	34.2

TABLE VI

RNA MONONUCLEOTIDES OF PAUL'S SCARLET ROSE STEM TISSUE CULTURES AT FIVE STAGES OF GROWTH, AND OF LEAF AND STEM TISSUES

Mononucleotide	μM/culture	μM/100 mg., alc. insol. residue	μM/g., dry wt.	Base ratio
8-day culture				
CMP	0.244	1.63	4.00	0.34
AMP	0.265	1.77	4.34	0.37
GMP	0.540	3.60	8.82	0.75
UMP	0.717	4.78	11.73	1.00
Total	1.766	11.78	28.89	
15-day culture				
CMP	0.245	0.59	2.18	0.30
AMP	0.287	0.69	2.55	0.43
GMP	0.469	1.13	4.18	0.70
UMP	0.668	1.61	5.93	1.00
Total	1.689	4.02	14.84	
22-day culture				
CMP	0.210	0.36	1.22	0.26
AMP	0.297	0.51	1.73	0.37
GMP	0.600	1.03	3.49	0.75
UMP	0.804	1.38	4.69	1.00
Total	1.911	3.28	11.13	
43-day culture				
CMP	0.280	0.26	1.19	0.27
AMP	0.237	0.22	0.97	0.23
GMP	0.602	0.56	2.25	0.58
UMP	1.033	0.96	4.48	1.00
Total	2.152	2.00	8.89	
Leaves				
CMP	—	4.22	24.75	0.60
AMP	—	4.54	26.65	0.64
GMP	—	5.43	31.90	0.77
UMP	—	7.07	41.50	1.00
Total	—	21.26	124.80	
Stems				
CMP	—	0.08	0.86	0.28
AMP	—	0.11	1.18	0.38
GMP	—	0.15	1.67	0.55
UMP	—	0.28	3.07	1.00
Total	—	0.62	6.78	

onate, although it was present in the citrate fraction of all tissue cultures (Table IV).

As the tissue cultures aged, free sugars increased to a maximum level at 29 to 43 days (Fig. 6, mg. per culture). The dominant sugar of the cultured tissue was sucrose, and there was generally slightly more glucose than fructose. Both stem and leaf tissues were much lower in sugars than the tissue cultures (Table V, mg. per g. dry weight). In stems, the major free sugars were glucose and fructose, with traces of sucrose and xylose present. Leaves were similar, except that they contained a considerable amount of sucrose.

The concentration of individual mononucleotides of the ribonucleic acid (RNA) of tissue cultures did not change appreciably during growth (Table VI), with one exception. It appeared that the concentration of uridylic acid in the RNA was greater in older tissues. This, of course, altered the base ratios of the mononucleotides at 43 days (Table VI). There was a close similarity between base ratios of stem and 43-day-old stem tissue culture RNA at 43 days. Leaf RNA, however, was found to be richer in cytidylic and adenylic acids. Total RNA per culture increased slightly during the 43-day experiment. On a dry weight basis (Table VI), there was a decrease in RNA which corresponded with increasing age. The greatest change occurred between the 8- and 15-day harvests, with a reduction in RNA of about 50 per cent. Stem tissues were relatively low in RNA, as expected, with the total level approaching that of 43-day-old tissue cultures. Leaf tissues, however, were found to contain from 4.3 to 14.1 times as much RNA as stem tissue cultures, and 18.5 times as much as stems.

Deoxyribonucleic acid (DNA) increased throughout the tissue culture growth period (Table VII). On a dry weight basis, however, DNA reached a maximum at 22 days, then fell off (Table VII). RNA/DNA ratios (Table

TABLE VII

DNA CONTENT OF PAUL'S SCARLET ROSE STEM TISSUE CULTURES AT FIVE STAGES OF GROWTH, AND OF LEAF AND STEM TISSUES

Sample	μG. DNA/ culture	μG. DNA/100 mg., alc., insol. residue	μG. DNA/g., dry wt.	RNA/DNA
8-day culture	53.5	357.0	874.0	11.2
15- “ “	109.1	263.0	972.0	5.2
22- “ “	215.0	370.0	1,254.0	3.0
29- “ “	228.0	297.0	1,087.0	—
43- “ “	259.0	240.0	1,059.0	2.8
Leaves	—	230.0	1,351.0	30.1
Stems	—	120.0	1,326.0	1.7

VII) of the tissue cultures dropped sharply between 8 and 15 days, and then declined gradually. The decrease in ratio was due primarily to the greater increase in DNA than in RNA during the growth period. Both leaves and stems contained about the same amount of DNA (Table VII, μg. per g. dry weight), and this quantity was found to be somewhat higher than those found for stem tissue cultures.

DISCUSSION

Paul's Scarlet rose stem tissue cultures undergo primarily catabolic reactions after being in culture for 3 to 4 weeks. These reactions occur in spite of the fact that the cultures were still growing (expressed as fresh or dry weights, Fig. 1). In some respects, the changes in composition with increasing age resemble those of attached leaves (8) or fruits (3). However, even though analogies may exist between the tissue cultures and attached plant parts, a true comparison cannot be made since the presence of a material in a plant part depends to a great extent upon the metabolic processes of other portions of the plant and the plant as a whole.

A number of interesting observations can be made, however. There is a decrease, due apparently to utilization, of free amino acids, bound amino acids, and organic acids after 3 or 4 weeks in culture. This sharp loss is not apparent in the free sugar fraction or in the RNA and DNA fractions. The results suggest, then, that in the presence of an adequate sugar supply and continuing nucleic acid synthesis in the tissues, there is a preferential loss of other soluble metabolites. It is possible that under the control of some cellular mechanism, senescence is triggered and then continues autonomously.

The level of RNA per culture after 8 days of growth is surprisingly high in light of the results for the subsequent 5 weeks. The over-all increase in RNA over the entire growth period was 12 per cent, while for DNA it was 485 per cent. Thus RNA increased only slightly during growth, while DNA increased sharply for 3 weeks, and gradually thereafter. The RNA/DNA ratio fell markedly between 1 and 3 weeks of growth. This decrease was correlated with a large number of nuclear divisions up to about 2 weeks. As the DNA level became relatively constant, nuclear divisions all but ceased.

Earlier investigations have shown that free amino acids of coconut milk are dominated by the presence of alanine and γ-aminobutyric acids (16). In Figure 2, it can be seen that in the tissue culture these two amino acids, unlike most others present, do not increase during the growth period, but decline rapidly after 8 days. This occurs in spite of the fact that a relatively large amount of these two acids (about 3 mg. per culture) is available in the growth medium.

One of the major free amino acids found in the tissue cultures was hydroxyproline (Fig. 3). The peak concentration of this amino acid occurred at 3 weeks, after which it fell off sharply. Hydroxyproline and proline were the only two amino acids of the proteins which increased throughout the growth period and were not affected by senescence. These results confirm the report that hydroxyproline is a major constituent of rapidly-growing, randomly-proliferating tissues (12). Dougall and Shimbayashi (2) and Lamport and Northcote (4), however, have shown that hydroxyproline is associated with a protein in cell walls even in mature tissues. The increase in bound hydroxyproline even after growth had slowed down is probably correlated with an increased amount of cell wall material.

Although some similarities exist between the stem tissue cultures and mature stem tissues of the rose, it can be generally concluded that the results support previous findings (17, 20) that tissue cultures are cytologically, physiologically, and biochemically distinct from the parent plant part.

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CHANGES IN FREE AMINO ACID AND AMIDE LEVELS OF LEAF PIECES, DETACHED LEAVES, DETACHED PLANTS, AND INTACT PLANTS OF TOBACCO AT DIFERRENT TIMES[1]

LEONARD H. WEINSTEIN AND CLARK A. PORTER

SUMMARY

Leaf pieces, detached leaves, detached plants, and intact plants of Vamorr 48 tobacco (*Nicotiana tabacum* L.) were placed in half-strength nutrient solutions for 24 and 72 hours. At each harvest, the different tissues were exposed to $C^{14}O_2$ in the light for 150 minutes, and the amino acid and amide contents, both labeled and nonlabeled, were compared with intact plants taken at zero time. For the most part, free amino acids and amides increased more in leaf pieces, detached leaves, and detached plants than in intact plants after 24 and 72 hours. This was especially true for amides. Asparagine always increased more than glutamine. Specific activities of the total amino compounds decreased in all tissues from the initial harvest at zero time to the 24-hour and 72-hour harvests, but in view of a considerable increase in amino compounds the actual quantities of C^{14} incorporated into amino compounds was higher in some instances. Highest specific activities of amino compounds were found in intact plants.

In general, there appears to be no advantage in the use of detached leaves or detached plants in preference to leaf pieces as a source of experimental material. However, it is suggested that intact plants are best adapted for many metabolic studies, including those associated with nitrogen metabolism and virus synthesis.

INTRODUCTION

There is often a tendency in physiological and biochemical experiments with plants, to use plant parts when most convenient. These experiments, many of a time-course nature, often last for several days, and may be complicated by microbial contamination. Results of these experiments are then often interpreted to explain the biochemical functions and mechanisms of intact growing plants. Experiments of this type are prevalent in studies of plant hormones and hormone action, mechanisms of action of toxicants, studies in plant virus synthesis and antiviral agents, and so forth.

It has been well established that the detachment of leaves or mutilated parts of leaves from plants quickly predisposes the tissue to a series of degradative changes. For example, proteolysis is almost immediately initiated (1, 4, 5, 6, 8, 9, 10). This is followed by an accumulation of free amino acids and amides, most of which are derived from proteolysis. A portion of the amides accumulated, however, is believed to be derived from a secondary origin (6, 9). Normal functions are destroyed because the vari-

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ous tissues are interdependent with each other. Nevertheless, this situation is often ignored in experimental studies.

Prior to a series of studies on the mechanism of action of 2-thiouracil on cucumber mosaic virus in tobacco plants (2, 3), time-course experiments were carried out with leaf pieces, detached leaves, detached plants, and intact plants in order to study changes in the concentration of free amino acids and amides, and the capability of the tissues to fix $C^{14}O_2$ in the light. The method used of expressing data on a per plant basis has been discussed earlier (2). The results of this experiment were sufficiently convincing that it was decided that intact plants should be used.

MATERIALS AND METHODS

A series of Vamorr 48 tobacco plants (*Nicotiana tabacum* L.) growing in nutrient culture (2) were selected for uniformity. Leaves removed from six plants were cut into 1-inch squares and floated on the surface of one-half strength nutrient solution (2). Leaves were removed from six other plants, and the lower portion of the petioles were placed in the nutrient solution. Roots were removed from six plants and the stems were placed in one-half strength nutrient solution. Six intact plants were transferred to fresh one-half strength nutrient solution. All of these materials were then maintained under prevailing diurnal light-dark conditions. As soon as possible, two intact plants were exposed to $C^{14}O_2$ in a bell jar in the light (7) for 150 minutes. Leaf tissues were then killed in boiling 80 per cent ethanol. After 24 and 72 hours, leaf pieces and detached leaves from two plants, and two detached plants and intact plants were exposed to $C^{14}O_2$ for 150 minutes. Methods used for the extraction of free amino acids and amides and their determination by two-dimensional paper chromatography have been described (3). Autoradiograms of the two-dimensional chromatograms were prepared with Kodak No-Screen X-ray film, and radioactive areas of the chromatograms were counted directly.

RESULTS AND DISCUSSION

For the most part, individual free amino acids and amides increased more in leaf pieces, detached leaves, and detached plants than in intact plants after 24 and 72 hours (Table I). This was perhaps most striking for asparagine and glutamine. The increases in total amino acids from zero time to 24 and 72 hours in leaf pieces were 1.3- and 2.2-fold, respectively; for detached leaves they were 1.5- and 3.2-fold; for detached plants, 1.3- and 4.2-fold; and for intact plants, 0.5- and 1.2-fold. The amide content of leaf pieces was 2.7 and 10.3 times larger, in detached leaves 1.6 and 8.3 times larger, in detached plants 3.3 and 8.1 times larger, and in intact plants 0.7 and 3.5 times larger than in intact plants at the initial harvest. Both asparagine and glutamine accumulated appreciably in leaf pieces,

TABLE I

CHANGES IN AMINO ACID AND AMIDE CONTENTS OF TOBACCO LEAF MATERIAL CULTURED IN NUTRIENT SOLUTIONS, SUBSEQUENT INCORPORATION OF C^{14} FROM $C^{14}O_2$ INTO THESE COMPOUNDS, AND DRY WEIGHTS OF TISSUES. VALUES EXPRESSED AS MG. PER PLANT

	0 Hours	24 Hours				72 Hours			
	Intact plants	Leaf pieces	Detached leaves	Detached plants	Intact plants	Leaf pieces	Detached leaves	Detached plants	Intact plants
Alanine	0.98	1.61	1.63	1.82	0.88	1.92	2.39	3.69	1.24
Arginine	0.59	1.33	0.87	1.22	0.45	3.16	3.23	4.32	2.93
Aspartic	2.21	5.72	4.94	2.29	3.15	3.67	4.88	10.6	4.36
γ-Aminobutyric	1.86	2.95	3.72	3.61	2.78	3.95	6.55	3.55	1.90
Glutamic	0.42	1.94	2.21	1.29	0.56	2.19	2.58	5.39	3.11
Glycine	0.15	0.42	0.35	0.46	0.22	0.53	0.61	0.82	0.40
Leucines	1.42	3.39	4.32	4.57	2.27	6.65	7.35	10.7	3.20
Lysine	0.36	1.31	1.21	1.02	0.54	1.41	2.06	2.83	1.04
Methionine sulfoxide	—	0.37	0.41	0.68	0.57	1.20	1.73	2.30	0.86
Phenylalanine	1.41	3.01	3.88	3.54	2.19	1.79	3.40	3.66	2.36
Proline	0.87	1.03	0.95	0.95	0.83	1.00	1.58	1.55	0.39
Serine	0.82	2.37	2.04	2.08	0.99	3.46	4.32	4.19	1.44
Threonine	0.94	1.79	1.80	2.28	1.70	2.26	3.09	4.00	1.84
Tyrosine	0.27	1.03	1.14	1.57	0.60	3.72	5.76	3.63	2.56
Valine	0.63	1.59	1.91	2.31	1.04	4.06	4.75	5.91	1.65
Asparagine	2.53	11.1	7.49	13.8	5.81	30.0	25.7	27.0	14.3
Glutamine	1.37	3.37	2.67	3.09	0.84	14.1	10.5	8.38	3.34
Total amino acids	12.93	29.86	31.38	29.69	18.77	40.97	54.28	67.14	28.47
Total amides	3.90	14.47	10.16	16.89	6.65	44.10	36.20	35.38	17.64
Total amino N	1.93	4.99	4.60	5.13	2.86	9.79	10.59	12.09	5.63
Total amide N	0.40	1.49	1.05	1.76	0.69	4.54	3.74	3.66	1.84
Specific activity of total (c.p.m./mg.)*	9,350	5,960	4,950	6,660	8,030	3,170	3,420	2,630	5,110
Dry wt. of leaves per plant (g).	1.37	1.43	1.36	1.68	1.42	1.35	1.61	1.70	1.40

* Based upon amino acids and amides which were found to be radioactive.

detached leaves, and detached plants. The percentage of total amino compounds was consistently larger for asparagine than for glutamine. This is in contradiction to results obtained by other workers for detached tobacco leaves cultured under somewhat similar conditions (5). When the concentrations of individual amino acids and amides were expressed as percentage of total constituents (Table II), glutamic acid made up a smaller percentage of the total in the detached plant and the intact plant at 24 hours than in leaf pieces or detached leaves. At 72 hours, however, the relationship changed inversely. The percentage of total amino compounds contributed by amides at 24 hours was higher in leaf pieces and detached plants than in detached leaves or intact plants. At 72 hours, amides made up over 50 per cent of the total amino compounds in leaf pieces, while in other treatments the value was 40 per cent or less. For the most part, the lower percentage of the total contributed by amides in intact plants at 72 hours was due to a lower amount of glutamine and not of asparagine. Asparagine was proportionately high in all tissues. Although asparagine contributed a higher percentage to the total nitrogen pool than glutamine, the accumulation of glutamine from 24 to 72 hours was relatively higher than asparagine (Table II).

After 72 hours of culture, the following amino acids and amides were labeled photosynthetically from $C^{14}O_2$: alanine, γ-aminobutyric acid, aspartic acid, glutamic acid, glycine, phenylalanine, serine, threonine, asparagine, and glutamine. The specific activities of the total amino acids and amides are shown in Table I. Specific activities from the initial harvest decreased in all tissues with increasing time. This was due primarily to the increase in soluble amino compounds in all tissues at all ages. Intact plants maintained the highest specific activities in the free amino acid and amide pool.

TABLE II

CHANGES IN AMINO ACID AND AMIDE CONTENTS OF TOBACCO LEAF MATERIAL CULTURED IN NUTRIENT SOLUTIONS, INDIVIDUAL AMINO ACIDS AND AMIDES EXPRESSED AS PER CENT OF TOTAL

	0 Hours	24 Hours				72 Hours			
	Intact plants	Leaf pieces	Detached leaves	Detached plants	Intact plants	Leaf pieces	Detached leaves	Detached plants	Intact plants
Alanine	5.9	3.6	3.9	3.9	3.5	2.3	2.6	3.6	2.7
Arginine	3.5	3.0	2.1	2.6	1.8	3.7	3.6	4.2	6.4
Asparagine	15.0	25.0	18.0	29.6	22.9	35.3	28.4	26.3	31.0
Aspartic	13.1	12.9	11.9	4.9	12.4	4.3	5.4	10.3	9.5
γ-Aminobutyric	11.1	6.7	9.0	7.8	10.9	4.6	7.2	3.5	4.1
Glutamic	2.5	4.4	5.3	2.8	2.2	2.6	2.9	5.3	6.7
Glutamine	8.1	7.6	6.4	6.6	3.3	16.6	11.6	8.2	7.2
Glycine	0.9	0.9	0.8	1.0	0.9	0.6	0.7	0.8	0.9
Leucines	8.4	7.7	10.4	9.8	8.9	7.8	8.1	10.4	6.9
Lysine	2 1	3.0	2.9	2.2	2.1	1.7	2.3	2.8	2.3
Methionine sulfoxide	—	0.8	1.0	1.5	2.2	1.4	1.9	2.2	1.9
Phenylalanine	8.4	6.8	9.3	7.6	8.6	2.1	3.8	3.6	5.1
Proline	5.2	2.3	2.3	2.0	3.3	1.2	1.7	1.5	0.8
Serine	4.9	5.4	4.9	4.5	3.9	4.1	4.8	4.1	3.1
Threonine	5.6	4.0	4.3	4.9	6.7	2.7	3.4	3.9	4.0
Tyrosine	1.6	2.3	2.7	3.4	2.4	4.4	6.4	3.5	5.6
Valine	3.7	3.6	4.6	5.0	4.1	4.8	5.2	5.8	3.6
Amides as % of total	23.1	32.6	24.4	36.2	26.2	51.9	40.0	34.5	38.2

Results of this experiment demonstrate that leaf punches, detached leaves, and detached plants each undergo degradative processes typical of starving detached leaves. There appears to be no advantage to the use of detached leaves or detached plants in preference to leaf pieces as a source of experimental material. Removal of roots from tobacco plants seems to eliminate an important regulatory influence which is not supplied by the presence of the stem. It is concluded that for certain types of experiments, especially those involved with nitrogen metabolism or virus synthesis, intact plants should be used wherever possible.

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THE EFFECTS OF MANGANESE UPON THE FREE AMINO ACIDS AND AMIDES OF THE TOMATO PLANT[1]

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SUMMARY

The influence of manganese upon the soluble amino acids and amides of the tomato (*Lycopersicon esculentum* Mill.) plant has been studied by use of two-dimensional paper chromatography and quantitative estimation of the nitrogen compounds by the ninhydrin reaction.

The major effects of manganese in tomato leaves were found to be:

(a) Manganese deficiency in the presence of either nitrate or ammonium-nitrogen produced an accumulation of soluble nitrogen compounds which consisted mainly of the amides, the accumulation from ammonium being much greater than from nitrate.

(b) A rapid decrease in the level of soluble nitrogen compounds (especially the amides) occurred when manganese was restored to plants which had become manganese deficient while they received adequate nitrate or ammonia.

(c) Restoration of manganese and nitrate to plants rendered deficient in both of these nutrients resulted in a rapid formation of soluble nitrogen compounds (mainly the dicarboxylic amino acids and glutamine). This synthesis was most rapid when both manganese and nitrate were furnished.

(d) The effects attributable to manganese are, however, complex; they cannot be interpreted in terms of a single and invariable effect, for they interact with factors of environment, nutrition, and development to which reference is made in the text.

(e) Manganese seems not to be implicated in the reduction of nitrate, nor in its immediate conversion to organic form, but it is conducive to the more rapid build-up of soluble nitrogen compounds, because it governs the supply of carbon acceptors for nitrogen via the Krebs cycle.

(f) In addition to the effects of manganese upon the mobilization of carbon at "ports-of-entry" for nitrogen into organic combination, the main seat of the effect of manganese on nitrogen metabolism is on the conversion of soluble products to protein as manganese acts to determine growth and protein synthesis. In this role interactions with the environment are conspicuous.

INTRODUCTION

This paper, which is the second in a series describing the influence of nutrition upon the soluble nitrogen compounds of tomato (*Lycopersicon esculentum* Mill.) plants, deals with the effects of manganese. The first paper (9) described the experimental procedures, the range of effects attributable to changes in the environment, and the effects due to in-

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organic nitrate and ammonium as nitrogen sources upon the nitrogenous compounds of tomato plants which were necessarily grown under somewhat fluctuating greenhouse conditions. Ammonium nutrition resulted in a high level of amide nitrogen; this contrasted with the effect of nitrate which produced a high level of dicarboxylic amino acid nitrogen. Plants that received a similar nutrient medium but were grown at different times in the fluctuating environment of the greenhouse responded with altered nitrogenous composition. The probable environmental factors which are of importance in this work were indicated, and their range of influence upon such metabolic effects as may be affected by the trace elements were analyzed.

The third paper in this series (14) describes the effects of molybdenum upon the soluble nitrogenous compounds of the tomato, and later it may be necessary to prepare a general synthesis of the results in relation to suggested interpretations of the functions of these trace elements in plant metabolism.

When this work commenced, there were strong suggestions in the literature that manganese was vitally involved in the nitrogen metabolism of higher plants. Manganese had been implicated in nitrogen metabolism by various authors; first in nitrate absorption and reduction (1) and later in ammonium assimilation into amino nitrogen and in protein synthesis (10). Important enzymes which mediate organic acid metabolism were also known to be influenced by manganese (11). Manganese is also considered to have some role in photosynthesis (3) and to affect the metabolism of green algae at many points [for summary see Gaffron (2), p. 183]. All of these postulated functions of manganese could have implications for nitrogen metabolism. Even so, the comprehensive account of nitrogen metabolism in plants (13), published as Vol. VIII of the Encyclopedia of Plant Physiology, edited by W. Ruhland, refers to very few specific effects of manganese. On page 214 it is recorded that nitrite and hydroxylamine reductase of soybean is influenced by manganese and, in consequence, manganese is credited with facilitating the reduction of nitrite to ammonia under relatively anaerobic conditions. Whereas, in the experiments of Hewitt, Jones, and Williams (7), nitrate accumulated in molybdenum deficient plants, manganese was deemed more essential for the conversion of amino acids to protein. It was, therefore, considered necessary to determine, quantitatively, the fluctuations in the soluble amino acids and amides in response to manganese and to the form of inorganic nitrogen applied.

EXPERIMENTAL RESULTS

The early indications of manganese deficiency in tomato are a mild interveinal chlorosis without an apparent effect upon growth. As the deficiency progresses, the chlorosis becomes more widespread, the plants become stunted, and a pin-point necrosis appears. The necrosis rapidly becomes the dominant characteristic, effects on growth are apparent, and the plants soon wither and die. For descriptions of the visible symptoms of manganese deficiency in various plants, reference may be made to Wallace (16) and to an account by Hewitt in the press to which the authors have had prior access (6). The experiments reported here involved the use of plants which exhibited the early stages of chlorosis. An objective in these experiments was to determine the effects of manganese upon nitrogen metabolism prior to the most adverse and extreme, or perhaps irreversible, effects of deficiency. The appearance of tomato leaves which exhibit mild or severe symptoms of manganese deficiency as they were developed in this work may be seen in Figure 1.

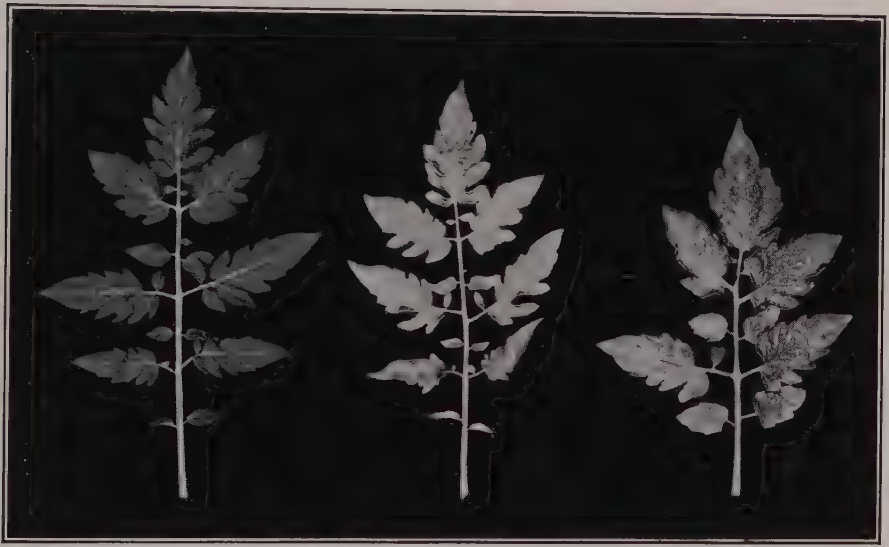

FIGURE 1. Typical leaves from tomato manganese deficiency experiments. Left to right: control, mild deficiency, severe deficiency.

The experiments as performed provided plants which exhibited different levels of manganese deficiency in plants grown with either nitrate or ammonium, and the effects of the deficiency are reflected in the altered composition of the soluble nitrogen fraction. Some data which are typical for leaves are presented in Table I. Amino acids which were detected, but present only in very small amounts, have been omitted from the tables.

The major effect of manganese deficiency upon the leaves of plants supplied with either form of nitrogen was to produce an accumulation of soluble nitrogen. This occurred even though other factors greatly influenced the level of the total soluble nitrogen in leaves in the different experiments. It was found repeatedly that manganese deficiency, in the presence of either form of nitrogen, and especially in the earlier stages of the deficiency, is associated with an accumulation of the amides. However, plants which were supplied with nitrate accumulated relatively more of their soluble nitrogen as dicarboxylic amino acids. With minor exceptions, the remaining components were usually but little affected by the presence or absence of manganese. In the nitrate treatments illustrated in Table I the disparities in the data for aspartic acid, serine, and glycine may be related to the effects of light which were discussed earlier (9). The measured solar radiation in experiment A was much higher than in experiment B. While no actual data for solar radiation are available for experiments C and D, an effect due to the environment may be inferred because there was twice as much lysine and three times as much arginine in the manganese deficient plants, compared with the controls in series C, while these amino acids were not present free in series D.

TABLE I

DISTRIBUTION OF NITROGEN IN THE PREDOMINANT SOLUBLE AMINO COMPOUNDS IN THE LEAVES OF TOMATO PLANTS GROWN WITH NITRATE OR AMMONIUM NITROGEN AND IN THE PRESENCE OR ABSENCE OF MANGANESE (NITROGEN IN EACH COMPOUND EXPRESSED AS μG. PER G. FRESH WEIGHT OF TISSUE)

Expt.	Nutritional treatment	Aspartic acid	Glutamic acid	Serine	Glycine	Asparagine	Threonine	Alanine	Glutamine	γ-Aminobutyric acid	Total nitrogen in ninhydrin reactive compounds
A	NO_3 plus Mn	53	25	17	24	0	0	12	24	7	162
	NO_3 minus Mn	29	49	10	0	44	9	13	48	15	217
B	NO_3 plus Mn	9	27	6	4	0	5	7	7	6	71
	NO_3 minus Mn	40	67	11	4	35	11	13	42	18	241
C	NO_4 plus Mn	0	8	41	0	304	0	0	455	5	894
	NO_4 minus Mn	0	20	72	0	302	0	0	774	9	1,380
D	NO_4 plus Mn	33	60	16	11	117	0	20	169	33	458
	NO_4 minus Mn	29	52	13	0	552	0	17	152	28	842

The effects of manganese deficiency on the concentration of the soluble amino acids and amides are related also to the severity of the symptoms and are complicated by the effect of age and of environment. Table II illustrates the results which were obtained on samples that exhibited different stages of deficiency. In an experiment (A) in which the deficient plants had a relatively low level of soluble nitrogen (lacking asparagine nitrogen entirely), the more extreme the deficiency the smaller this pool of soluble nitrogen became. In another experiment (B), in which the pool of soluble nitrogen in the initially deficient plants was much greater, containing both amides asparagine and glutamine, the further deficiency caused a less dramatic fall in the soluble nitrogen which was mainly in the amide asparagine, aspartic and glutamic acids. The patterns in the concentrations of the individual components are rather complex and are doubtless due to interactions of the effects of the trace element with the effects of environmental factors and age of the plants upon the metabolism of amino acids.

The background against which the effects attributable to manganese may be interpreted must of necessity be the range of variation in the size and composition of the pool of soluble nitrogen compounds which may be encountered even in plants grown in a full nutrient solution, with nitrogen furnished either as nitrate or ammonium in the greenhouse. This information was contained in the first paper of this group, and it is summarized as follows:

TABLE II

DISTRIBUTION OF NITROGEN IN THE PREDOMINANT SOLUBLE AMINO COMPOUNDS IN THE LEAVES OF TOMATO PLANTS GROWN WITH NITRATE AND EXHIBITING DIFFERENT DEGREES OF MANGANESE DEFICIENCY (NITROGEN IN EACH COMPOUND EXPRESSED AS μG. PER G. FRESH WEIGHT OF TISSUE)

Expt.	State of Mn deficiency	Aspartic acid	Glutamic acid	Serine	Asparagine	Threonine	Alanine	Glutamine	γ-Aminobutyric acid	Total nitrogen in ninhydrin reactive compounds
A	Initial stage	23	53	0	0	0	14	25	10	139
	Advanced stage: chlorotic	15	29	7	0	0	4	5	5	65
	Necrotic	25	37	0	0	0	12	6	6	90
B	Initial stage	53	65	4	81	0	12	31	4	249
	Advanced stage: necrotic	29	49	10	44	9	13	48	15	217

(1) Under otherwise comparable conditions, nitrate nutrition produces a lower content of soluble nitrogen compounds in the leaves than when ammonium is supplied; it emphasizes dicarboxylic acid nitrogen in preference to amide nitrogen; and it emphasizes glutamine in preference to asparagine.

(2) By contrast, ammonium nutrition leads to a much higher content of soluble nitrogen and a heavy accumulation of amide nitrogen over dicarboxylic acid nitrogen, with asparagine as the less conspicuous amide under full nutrient conditions.

(3) If the seasonal and other variables cause the plants to accumulate various levels of soluble nitrogen in their leaves, as they may do over as much as a sixfold range, then, provided the plants receive full nutrient solution with nitrate as the source of nitrogen, the characteristics of the "nitrate-nitrogen" type of nutrition persist. Therefore, glutamine-nitrogen, glutamic acid nitrogen, alanine, and γ-aminobutyric acid all tend to vary *pari passu* with the total soluble nitrogen of the leaves.

Similarly, if the conditions that prevail during growth greatly alter the total soluble nitrogen of the leaves of ammonia-nitrogen, full nutrient plants they do so in such a way as to preserve the essential characteristics of this type of plant. These characteristics are a large relative accumulation of total amide nitrogen, i.e., glutamine and asparagine nitrogen, with glutamine in excess, although there is still room for unexplained variations in the proportion of asparagine to glutamine due to the conditions that prevail during growth.

(4) Furthermore, when otherwise full nutrient plants which have been deprived of nitrate-nitrogen as their only source of nitrogen until they have become nitrogen-deficient, and have in consequence a low soluble nitrogen content, are allowed to recover in a renewed nitrate supply, then the increase of soluble nitrogen in the leaves is predominantly in the form of glutamine.

(5) Roots, however, behave somewhat differently, although the range of response of the total soluble nitrogen under external conditions is not dissimilar to that in leaves. However, asparagine is conspicuous here, and is even comparable to glutamine in full nutrient nitrate-nitrogen plants, and the basic amino acids (arginine and lysine) which are virtually absent in the free state from the full nutrient leaves may be conspicuous in their roots. (The occurrence of asparagine-nitrogen, arginine, and lysine in the soluble nitrogen of tomato roots which are exposed to nitrate containing cultures is indicative that these substances may be re-exported from leaves and thus may be more representative of the mobile breakdown products from leaves than they are indicative of the products formed directly from entering nitrate-nitrogen.) The uncontrolled factors during the growth of plants in the greenhouse, during a protracted period of time, permitted the soluble nitrogen content of tomato roots to vary in different crops over a fourfold range, but the relative composition with respect to the principal constituents named remained relatively steady. For example, γ-aminobutyric acid, arginine, lysine, glutamine, alanine, asparagine, and glutamic and aspartic acids in their nitrogen content remained positively correlated with the total soluble nitrogen of the roots.

It would, therefore, be surprising if, against this background, the effects of manganese deficiency on the metabolism of whole tomato plants or of their leaves were to be a single and invariable effect. The interactions of the environmental effects, the severity of deficiency, the source of nitrogen, and the internal economy of the plant body as nitrogen is moved from one organ to another together produce a complex situation. However, the main effects which are attributable to the presence, or lack, of manganese may be diagnosed as follows.

Table I shows the effect of the presence or absence of manganese in the nitrate and ammonium types of nutrient upon the composition of the soluble nitrogen of tomato leaves. Data are given for two experiments;

these were experiments in which the uncontrolled factors had operated in such a way as to give different levels of soluble nitrogen in the leaves as indicated above. However, the first effects of manganese are clear:

(a) Whether the source of nitrogen was nitrate or ammonia, the lack of manganese usually caused a greater accumulation of soluble nitrogen than would otherwise have obtained; and this effect was independent of the factors that tended to regulate the level of total soluble nitrogen which was present in the leaves.

(b) Manganese lack, which caused the accumulation of soluble nitrogen, also caused the appearance of asparagine in tomato leaves grown on nitrate nitrogen, a condition under which it would not otherwise occur, and also an increase in glutamine. The plants grown on ammonium as the source of nitrogen were already high in total amide, even in the presence of manganese; this was substantially increased when manganese was omitted, though the increase could occur either in the form of asparagine or of glutamine as determined by factors that may differ from one experiment to another.

The conditions which permit the above somewhat clear-cut effects to be observed are those in which the deficient plants are still able to mobilize external sources of nitrogen and to convert them into the soluble form. Thus the main block caused by lack of manganese is here an ultimate interruption in protein synthesis. These are the conditions, therefore, in which soluble nitrogen tends to be high. If, however, the conditions of deficiency are so complicated by factors in the organs analyzed that the nitrogen reserves are not so mobilized and the cells, either through inability to cope with external nitrogen or by competition and loss to other regions, progressively lose organic nitrogen, then a seemingly different situation prevails. Under these latter circumstances, the more severe the deficiency the *lower* the total soluble nitrogen content. In these circumstances, results of the kind summarized at (c) below may be encountered.

(c) If the severity of the manganese deficiency is progressively increased, there is a point at which it appears to cause an actual *decrease* in the soluble nitrogen content of leaves, which, grown on nitrate without manganese, had become first chlorotic and then necrotic. The total soluble nitrogen under these circumstances, and also the principal constituent substances, may decrease with increasing severity of the conditions in the leaf. These are the data to be seen in Table II. These conclusions may also apply to such individual constituents as aspartic and glutamic acids as well as to the combined dicarboxylic amino acid nitrogen, and it may be true also of the total amide (asparagine+glutamine), even though the former substance, under certain cases of severe deficiency, may no longer be present.

Equally diverse effects attributable to manganese deficiency may be encountered in roots. In Table III are some data on the levels of soluble nitrogen compounds that develop in certain roots with various levels of manganese deficiency and which were supplied with either nitrate or ammonium. (These roots were unusually poor in products attributable to translocation from leaves such as asparagine, arginine, etc.) However, at all stages of the deficiency of manganese the roots of the deficient plants, which had received nitrate, had even *less* soluble nitrogen than those which received manganese. This contrasts with the most frequent data on leaves (Table I), since the main effect there was to cause the nitrogen compounds to accumulate under manganese deficiency. The leaves of manganese deficient plants are dominated by their failure to convert the soluble nitrogen they can form to protein; but the roots, which can clearly form soluble compounds which accumulate from ammonium sources even when the roots are manganese deficient, do so even less readily when they are deprived of manganese, if the source of nitrogen is nitrate.

TABLE III

DISTRIBUTION OF NITROGEN IN THE PREDOMINANT SOLUBLE AMINO COMPOUNDS IN THE ROOTS OF TOMATO PLANTS SUPPLIED WITH NITRATE OR AMMONIA (NITROGEN IN EACH COMPOUND EXPRESSED AS μG. PER G. FRESH WEIGHT OF TISSUE)

Expt.	State of deficiency	Nutrient treatment	Aspartic acid	Glutamic acid	Asparagine	Alanine	Glutamine	γ-Aminobutyric acid	Total nitrogen in ninhydrin reactive compounds
A	First symptoms	NO_3 plus Mn	8	21	0	0	15	11	67
		NO_3 minus Mn	0	5	0	12	8	2	27
	1 Week later	NO_3 plus Mn	4	9	8	5	15	8	65
		NO_3 minus Mn	6	19	0	2	0	4	34
	2 Weeks later	NO_3 plus Mn	2	12	11	2	9	7	45
		NO_3 minus Mn	0	7	0	0	25	4	36
B	Severe	NH_3 plus Mn	0	14	154	0	406	22	605
		NH_3 minus Mn	7	10	121	0	221	10	382
C	Severe	NH_3 plus Mn	8	14	199	3	128	16	395
		NH_3 minus Mn	11	14	483	0	203	0	727

Therefore, the diagnosis of the metabolic effects which are attributable to prolonged manganese deficiency is very complex. It depends on the source of nitrogen, i.e., whether nitrate or ammonium; it depends on the organ in question, i.e., whether leaves or roots. It also depends on certain subtle combinations of the environmental conditions which have prevailed during growth, to determine the kind of plant which is produced and whether it tends to accumulate asparagine or glutamine from ammonium sources when manganese is deficient; and also whether it responds to more acute manganese deficiency by an increase or a decrease in the soluble nitrogen content of the tissues. The common effect to be noted in all the various effects that have been described is that they are ultimately concerned with the facility with which organic nitrogen is utilized by the tissue in growth and protein synthesis compared with its ease of absorption and dispersal in the available storage forms.

This being so, another type of experiment was adopted in the hope that it would more directly point to the step which is immediately limiting the metabolism in the deficient plants. In this type of experiment, instead of observing the relatively long term accumulation of soluble nitrogen compounds during their growth, the *short term changes* were observed in experiments in which *the plants were allowed to recover from the deficiency in question.* Thus, manganese was supplied with, or without, nitrate or ammonium to plants which had been rendered deficient in either manganese or manganese and nitrogen. The hope was that the *first* discernible changes would reveal the most important step previously limited by lack of manganese.

TABLE IV

Distribution of Nitrogen in the Predominant Soluble Amino Compounds in the Leaves of Tomato Plants Recovering from Manganese and Nitrogen Deficiencies (Nitrogen in Each Compound Expressed as μG. per G. Fresh Weight of Tissue)

Treatment applied to plants		Concentration of ninhydrin reactive compounds					
Nutrient solution	Duration of treatment	Aspartic acid	Glutamic acid	Alanine	Glutamine	γ-Amino-butyric acid	Total amount of N determined
NO_3 plus Mn	6 hours	22	35	3	51	7	118
NO_3 minus Mn	6 "	18	24	5	46	9	102
NO_3 plus Mn	30 "	34	41	9	77	7	184
NO_3 minus Mn	30 "	9	21	8	49	5	107
Minus N plus Mn	6 "	6	12	0	0	7	29
Minus N minus Mn	6 "	7	14	2	0	6	34
Minus N plus Mn	30 "	8	14	3	6	7	44
Minus N minus Mn	30 "	8	8	0	0	2	18

In a group of experiments the recovery of tomato plants from manganese deficiency was investigated. The treatments involved the prior development of manganese deficiency followed by a period of lack of nitrogen; nitrogen was then supplied in the presence, or absence, of manganese. Table IV outlines the salient results of these experiments. The resupply of nitrate *alone* to nitrogen and manganese deficient plants permitted the synthesis of amino compounds, albeit to a lower level than the plants which received both components simultaneously. This effect was confined to aspartic and glutamic acids and, in particular, to glutamine. The data indicate that the manganese which was still available in the tissue permitted a certain level of synthesis of amino compounds to be achieved up to 6 hours after the start of the treatment. The treatments in which nitrogen deficiency was maintained establish the level of soluble compounds from which the rapidity of the recovery in this fraction, when nitrogen is restored, may be measured. The stationary levels of glutamine and glutamic acid between 6 to 30 hours in manganese deficient plants supplied with nitrate contrast with the increase of these compounds in leaves which were also recovering from manganese deficiency. Therefore, manganese promotes the formation of glutamic acid and glutamine even from nitrate as the source.

Information was also obtained from a study of the leaves of plants which were originally deficient in both manganese and nitrogen (Table V). These plants were subsequently provided with nitrate, and one-half of them received manganese for periods up to 30 hours. The data showed: (a) Manganese deficiency did not prevent the synthesis of soluble nitrogen compounds after the resupply of nitrogen in the form of nitrate. In fact in the earlier stages of recovery from nitrogen deficiency the soluble compounds were built up *faster* in the absence of manganese, presumably because when this was present recovery also included the conversion of the soluble compounds to protein. (b) Only after 30 hours, by which time the soluble compounds could have overtaken their depletion by protein synthesis, did the combined use of nitrate and manganese cause soluble compounds to equal or increase over that in the plants that remained manganese deficient.

TABLE V

CHANGES WITH TIME IN THE DISTRIBUTION OF NITROGEN IN THE PREDOMINANT SOLUBLE AMINO COMPOUNDS IN THE LEAVES OF TOMATO PLANTS ORIGINALLY DEFICIENT IN NITRATE AND MANGANESE (NITROGEN IN EACH COMPOUND EXPRESSED AS μG. PER G. FRESH WEIGHT OF TISSUE)

Treatment applied to plants		Concentration of ninhydrin reactive compounds							
Nutrient solution	Hours after addition of NO_3 and/or Mn	Aspartic acid	Glutamic acid	Asparagine	Alanine	Glutamine	Arginine	γ-Aminobutyric acid	Total amount of N determined
Mn and N deficient	0	4	4	27	8	16	11	24	109
NO_3 plus Mn	2	10	5	23	13	25	15	22	147
NO_3 minus Mn		9	8	26	21	22	27	28	199
NO_3 plus Mn	6	16	0	17	25	37	29	33	217
NO_3 minus Mn		17	0	25	26	44	27	40	248
NO_3 plus Mn	24	16	0	35	27	40	30	37	248
NO_3 minus Mn		13	0	24	26	38	31	33	227
NO_3 plus Mn	30	20	0	0	29	100	45	50	320
NO_3 minus Mn		24	0	30	29	97	27	49	302

Two series of experiments were made on the changes in roots which accompany the resupply of nitrate (with and without Mn) or ammonium (with and without Mn) to plants previously deficient in both nutrients. In one group of experiments, which had been rendered nitrogen and manganese deficient for 7 days, the plants showed but little response to the resupplied nutrients, at least in their roots, for in this experiment, no single constituent or even the total soluble nitrogen increased dramatically even up to 30 hours after the deficiencies were terminated. Generally the resupply of NO_3 with Mn gave a somewhat greater accumulation of soluble nitrogen than if manganese was still deficient, but no soluble compound increased dramatically, not even glutamine, when ammonium and manganese were added. In another experiment, however, in which the prior deficiencies of manganese and nitrogen were established over 6 days, there was a more impressive build-up of soluble compounds after recovery and this was most marked when ammonium was resupplied giving amounts of total soluble-nitrogen and of amide (mainly glutamine) which were *greater* when manganese was still deficient (because it limited protein synthesis).

Restoration of manganese to deficient plants, which had been cultured with an uninterrupted supply of ammonia, resulted in a rapid decline in the soluble nitrogen of leaves with this effect being localized in the amides, asparagine in particular (Table VI). In the leaves of plants grown with an uninterrupted supply of either ammonium or nitrate, a similar result was obtained—the effects of ammonium nitrogen reinforce the results with nitrate. Manganese deficiency usually resulted in an accumulation of soluble nitrogen, and the resupply of the trace element promptly reduced this, with a specially pronounced effect upon the amides. There appears, therefore, to be little effect of manganese upon the synthesis of amino acids from externally supplied nitrate or ammonium, or upon the ability of the tomato leaf to synthesize the carbon moieties of amino acids and amides.

TABLE VI

RECOVERY OF THE LEAVES OF TOMATO PLANTS SUPPLIED WITH AN UNINTERRUPTED SUPPLY OF AMMONIA FROM MANGANESE DEFICIENCY AS REFLECTED IN THE PREDOMINANT SOLUBLE AMINO COMPOUNDS (NITROGEN IN EACH COMPOUND EXPRESSED AS μG. PER G. FRESH WEIGHT OF TISSUE)

Nutrient treatment	Concentration of ninhydrin reactive compounds						
	Aspartic acid	Glutamic acid	Asparagine	Alanine	Glutamine	γ-Aminobutyric acid	Total amount of N determined
Mn deficient	29	52	552	17	152	28	842
Mn supplied for 24 hours	14	31	265	8	98	15	452

Plants which had been rendered both nitrogen and manganese deficient were analyzed at various intervals after the addition of ammonium. Considerable levels of soluble nitrogen were achieved even in leaves still deficient in manganese (Table VII); the concentration attained early

TABLE VII

EFFECTS OF MANGANESE AND AMMONIA UPON THE SOLUBLE NITROGEN COMPOUNDS OF TOMATO LEAVES AFTER A PERIOD OF MANGANESE AND NITROGEN DEFICIENCY (NITROGEN IN EACH COMPOUND EXPRESSED AS μG. PER G. FRESH WEIGHT OF TISSUE)

Expt.	Treatment applied to plants		Concentration of ninhydrin reactive compounds									
	Nutrient solution	Hours after addition of Mn and/or NH_3	As-partic acid	Glu-tamic acid	Serine	Glycine	Aspara-gine	Alanine	Gluta-mine	Argi-nine	γ-Amino-butyric acid	Total amount of N de-termined
1	Plus NH_3 and Mn	6	15	23	11	0	0	9	101	0	16	173
		30	10	27	127	24	26	22	378	0	39	652
	Plus NH_3 minus Mn	6	22	32	11	0	15	16	158	0	24	278
		30	12	23	47	6	51	16	167	0	26	348
2	N and Mn deficient	0	4	4	4	2	27	8	16	11	24	109
	Plus NH_3 and Mn	2	8	0	6	3	59	16	27	31	33	237
		6	14	0	7	4	62	23	51	38	31	293
		24	9	0	7	4	68	18	81	25	40	293
		30	14	0	9	5	94	24	140	45	41	427
	Plus NH_3 minus Mn	2	9	0	6	4	22	19	26	27	33	194
		6	13	0	6	3	23	16	38	19	31	176
		24	10	0	8	4	33	19	82	0	44	233
		30	13	0	9	6	46	18	173	11	36	351

(experiment 1, 6 hours) was greater than in plants supplied with manganese, but at 30 hours this position was reversed. In manganese deficient leaves, glutamine reached a peak concentration early, and thereafter remained constant. Restoration of manganese produced a rapid increase of glutamine, and at 30 hours it reached a level about 3.8 times greater than it was at 6 hours. Asparagine appeared more promptly in the manganese deficient leaves, and its level rose with the time of exposure to ammonium; in the presence of manganese a small quantity of asparagine was found only at the final harvest. Experiment 2 (Table VII) supported some of these results. Lack of manganese did not prevent the formation of a considerable amount of soluble nitrogen and especially glutamine. Far less asparagine was present at 30 hours in the deficient leaves than in those replenished with manganese. Up to 30 hours of exposure, less glutamine was detected in deficient leaves as the level of asparagine remained almost constant at the final harvest. An obvious interrelationship exists between the two amides, glutamine and asparagine. In experiment 1, leaves deficient in manganese had less glutamine but more asparagine than plants recovering from the deficiency; in experiment 2, the effects were reversed. The conditions which favor one or the other amide are not, as indicated above, precisely known.

A single experiment was performed to compare the response to manganese of tomato and other plants. Corn (*Zea mays* L.) was selected as the alternative plant for investigation. The response to manganese deficiency by corn leaves is dictated by the form of nitrogen supplied: less total amino nitrogen occurs in the presence of nitrate and an accumulation in the presence of ammonia (Table VIII). Corn and tomato leaves differ in the composition of the soluble amino acid pool. Corn leaves nourished by nitrate have no glutamine, γ-aminobutyric acid, or asparagine and relatively higher concentrations of alanine, serine, glycine, and the basic amino acids. Manganese deficiency, in the presence of nitrate, results in a decline in the concentrations of all the soluble amino acids except glycine and arginine. The response of corn leaves to ammonia-nitrogen in an

TABLE VIII

DISTRIBUTION OF NITROGEN IN THE PREDOMINANT SOLUBLE AMINO COMPOUNDS OF CORN GROWN WITH NITRATE OR AMMONIA AND IN THE PRESENCE OR ABSENCE OF MANGANESE (NITROGEN IN EACH COMPOUND EXPRESSED AS μG. PER G. FRESH WEIGHT OF TISSUE)

Nutritional treatment	Aspartic acid	Glutamic acid	Serine	Glycine	Asparagine	Alanine	Glutamine	Lysine	Arginine	Total nitrogen in ninhydrin reactive compounds
NO_3 plus Mn	31	51	73	37	0	52	0	21	27	310
NO_3 minus Mn	9	28	43	37	0	29	0	0	27	172
NH_4 plus Mn	16	23	80	0	242	79	29	25	48	553
NH_4 minus Mn	20	126	73	0	527	59	0	0	26	848

otherwise full nutrient medium is to synthesize asparagine, and manganese deficiency produces an accumulation of asparagine and glutamic acid. The glutamine seems to play a more minor role in corn than in the tomato plant. Thus a different pattern of enzymatic controlled and trace element directed reactions affecting the soluble amino compounds may well be indicated.

CONCLUSIONS

The evidence presented in this paper has again amply demonstrated, for tomato plants, that their manganese status exerts a considerable effect upon the level of the alcohol soluble nitrogenous compounds which they contain, i.e., notably the amino acids and the amides glutamine and asparagine.

In otherwise comparable cultures, Possingham (12) grew tomato plants and analyzed these for their nitrogenous constituents to compare the effects due to lack of iron, copper, manganese, and molybdenum. Although in Possingham's experiments other deficiencies (especially Fe, Ca, and Mn) produced massive accumulations of soluble nitrogen compounds, this was not so conspicuous in the case of manganese. In fact, the principal increases were in the dicarboxylic amino acids and the total amide (largely composed of asparagine in these plants) actually decreased with manganese deficiency. These earlier and limited data of Possingham (12), together with the different types of effects here reported, serve to stress that the effects attributable to manganese and exerted through the nitrogen metabolism are certainly not simple and direct, and they interact with other nutritional and environmental variables to produce an array of responses which are baffling if this fact is not appreciated. The complexity and multiplicity of the effects on plant metabolism due to manganese have been stressed (4, 5). Reference may also be made to a recent survey of these problems (6) which is now in press.

Apparently manganese is not specifically implicated in the reduction of nitrate or in its immediate conversion to an organic form, since this will proceed substantially in nitrate and manganese deficient plants on the resupply of nitrate alone. Manganese is, however, conducive to the more rapid build-up of soluble compounds from nitrate, especially glutamic acid and glutamine, presumably because it governs the supply of the carbon acceptors of, or the "ports-of-entry" for, nitrogen via the Krebs cycle.

The levels of soluble nitrogen compounds in the tomato, as these are affected by the exogenous sources, nitrate and ammonia on the one hand and manganese on the other, are governed by two opposing trends. These trends are: (a) the accumulation of the soluble compounds which are stored in excess of the conversion of nitrate reduction products, or of exogenous ammonium, to protein; (b) the utilization of soluble nitrogen compounds in growth and protein synthesis.

When synthesis of protein and growth proceeds normally, in the presence of manganese, from nitrate as the nitrogen source, the reduction of nitrate and the storage of soluble compounds does not outpace conspicuously the conversion to protein and the utilization in growth. In consequence, asparagine, which is distinctively a storage product, rather than a true synthetic intermediate in leaves, is not found; it does, however, occur in roots which may store compounds translocated from leaves. However, when growth and synthesis are arrested by lack of manganese, amides—and even asparagine—do accumulate, and when manganese is resupplied they also tend to disappear.

The most massive accumulation of soluble compounds, and especially amides including asparagine, occurs, however, when ammonium is the source; and no doubt much of the carbon which, with nitrate as the source of nitrogen, would have moved in a balanced fashion into protein is now, as it were, "drained off" from the carbohydrate metabolism by the need to convert the large amounts of absorbed ammonia into these soluble storage products. Again, when growth and protein synthesis are arrested by lack of manganese, the concentrations of the compounds formed from ammonia increase still further, and they are promptly reduced again if manganese is resupplied.

Thus the main seat of the effect of manganese on nitrogen metabolism is on the conversion of preformed soluble products to protein as it acts to determine growth and protein synthesis. Manganese obviously has effects also upon the mobilization of carbon through the Krebs cycle to form the "ports-of-entry" for nitrogen into organic combination with carbon. It is this aspect of metabolism which has been found to be sensitive to environmental and other interacting nutritional factors, at least in the case of mint (14), in ways that could determine the balance between dicarboxylic amino acids and their amides, the 4- and 5-carbon compounds, such as asparagine and glutamine, etc.

It is, therefore, not surprising that the body of data in this paper presents a number of problems of interpretation when the details of the composition of the soluble nitrogen pool as it is affected by manganese are considered. Other authors (4, 5, 6 and references there cited) have also recognized the complexity of this problem. However, having indicated the main kinds of effects upon the nitrogen metabolism of the tomato which are associated with manganese, any further discussion is deferred until the effects which are attributable to molybdenum have also been described, so that a more comprehensive summary with respect to both these trace elements may then be attempted.

While the data for corn show that manganese is also implicated in determining the levels of soluble nitrogen compounds that obtain in this plant they indicate that the consequences of manganese nutrition or deficiency may be quite different from the responses observed in the tomato. While ammonia nutrition leads to high soluble nitrogen content in the leaves, higher when manganese limits growth and protein synthesis, the principal amide here is asparagine though glutamic acid accumulated under nitrate nutrition. The levels of soluble nitrogen are much lower and both amides (asparagine and glutamine) were virtually absent and, with nitrate as the source, manganese promotes the soluble nitrogen compounds whereas its deficiency caused them to be at a lower level. Therefore, although manganese is intimately involved in the course of nitrogen metabolism, one cannot argue directly from tomato plants to corn plants for each has its metabolism idiosyncrasies.

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THE EFFECTS OF MOLYBDENUM UPON THE FREE AMINO ACIDS AND AMIDES OF THE TOMATO PLANT[1]

F. C. STEWARD[2] AND DAVID MARGOLIS[3]

SUMMARY

The influence of molybdenum, combined with nitrate or ammonium as the source of nitrogen, upon the predominant soluble amino acids and amides of the tomato (*Lycopersicon esculentum* Mill. var. Rutgers) was studied by methods based on quantitative paper chromatography.

Plants which had received a continual supply of nitrate nitrogen but which were deficient in molybdenum were characterized by a lower concentration of glutamine than plants rich in molybdenum, though certain amino acids were present in somewhat increased amounts in the younger leaves. Recovery from molybdenum deficiency in the presence of nitrate was accompanied by a temporarily increased level of soluble nitrogen, especially of glutamine.

Plants initially deficient in both molybdenum and nitrogen contained less soluble nitrogen, especially glutamine, and upon the addition of nitrate alone they responded less than comparable plants which received both nitrate and molybdenum, when their glutamine content increased prominently. The substitution of ammonia for nitrate eliminated any significant influence of molybdenum upon the soluble amino acids and amides of the tomato, for the plants achieved the high amide content of ammonia-grown plants irrespective of their molybdenum status.

In roots arginine and asparagine were more prominent than in leaves, and the latter, asparagine, was found to be responsive to molybdenum during recovery from its deficiencies in the presence of nitrate.

The data are interpreted in terms of the known role of molybdenum in nitrate reduction and on the basis that molybdenum, unlike manganese, does not specifically affect the subsequent course of nitrogen metabolism. Although quantitative effects on the usage of nitrogenous metabolites may result from effects of molybdenum upon total growth, quantitative effects that affect the course of metabolism and which interact with environmental factors that determine growth and development have not been encountered. In this respect manganese and molybdenum are different.

INTRODUCTION

Molybdenum has rapidly become important both in agriculture and in plant physiology. After the spectacular results achieved in the pastures of Australia (2), it has become common horticultural practice to consider the possible need for molybdenum in a fertilizer program. A symposium on the subject was held in 1956 (4).

The study of the essentiality and functions of molybdenum is relatively

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recent. In 1930 Bortels found that *Azotobacter* responded to molybdenum with an increase in nitrogen fixation (5). The first response of a higher plant to molybdenum was that demonstrated by Arnon and Stout in 1939 (3). The necessity of molybdenum for nitrogen fixation by clover and alfalfa was established in 1942 (1). As early as 1948, Stout and Meagher (15) had shown that the mottled areas in leaves of molybdenum deficient plants contained an excess of nitrate and that the accumulated nitrate disappeared when minute amounts of radioactive molybdenum were injected. Much effort has been expended in attempts to locate the precise site of molybdenum action in the reduction of nitrate. In 1954, molybdenum was shown to be an important factor in nitrate reductase (10), and Spencer and Wood (13) showed that when molybdenum was restored to molybdenum deficient tomato plants it first caused nitrite to increase (up to 2 hours) and later ammonia increased (after about 6 hours).

The story of molybdenum in plant metabolism is, however, not yet complete. Many investigations, ranging from Bortels (5) in 1930 to papers summarized by Hewitt (6) in 1962 have made reference to the role of molybdenum in nitrogen metabolism. However, the participation of molybdenum in enzymatic reactions, other than those immediately concerned with nitrogen metabolism, has also been suggested (12).

It is the purpose of this paper to present the results of experiments which were concerned with the influence of molybdenum upon the utilization of inorganic nitrate and ammonium salts by the leaves and roots of tomato plants. This paper, the third in this series, describes experiments which were designed to reveal how certain trace elements intervene in the events which govern the metabolism of amino acids and amides in higher plants.

EXPERIMENTAL RESULTS

Procedures to establish molybdenum deficiency in the experimental tomato plants will not be described in detail. They have been fully presented elsewhere (8), and in any event descriptions of these techniques and of the symptoms that result are now numerous. Hewitt and Jones (7) described the production of symptoms of molybdenum deficiency in tomato plants as early as 1947, and Hewitt has recently reviewed this field of work (6) with copious citations to the literature. The contrasted appearance of molybdenum deficient and full nutrient tomato plants, after they had been grown for use in these experiments in polyethylene containers, is shown in Figure 1.

Against the background and knowledge gained and reported in the earlier papers, four kinds of experiments were performed. These were to reveal the following effects of molybdenum:—

(1) The effects of molybdenum on the size and composition of the pool

of soluble nitrogen compounds of plants which had, during protracted growth with and without molybdenum, become adjusted to molybdenum deficiency as contrasted with a full nutrient supply. These observations were carried out mainly on leaves, but, inasmuch as the symptoms of molybdenum deficiency differ in the leaves of different ages, a distinction was made between leaves higher and lower on the axis.

(2) The effects on the size and composition of the pool of soluble nitrogen compounds of restoring molybdenum to molybdenum deficient plants, which continued to receive nitrate as their source of nitrogen. Again it was necessary to distinguish between younger and older leaves, and, in this type of experiment, the first responses to added molybdenum could be followed with time.

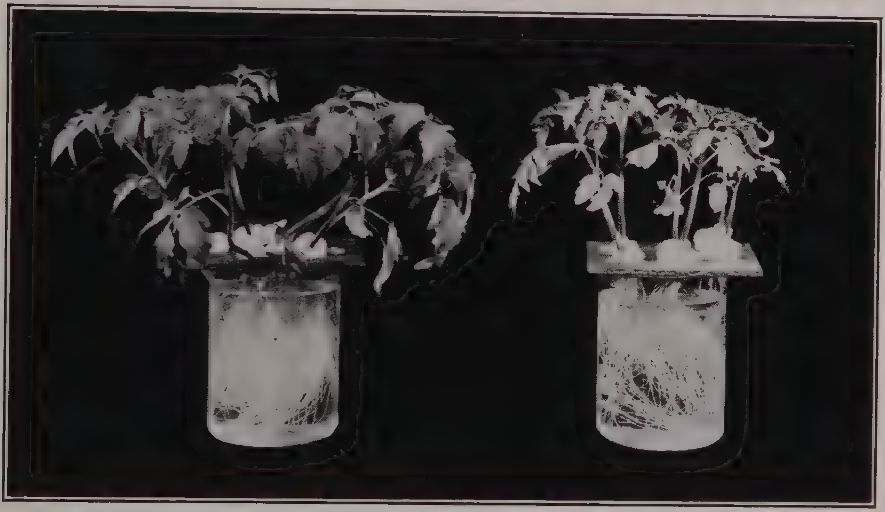

FIGURE 1. Plants from molybdenum deficiency experiments. Left control, right mild molybdenum deficiency. The aluminum foil used to exclude light from the roots during the experiments was removed to permit comparisons of root development.

(3) The effects of restoring molybdenum, either with or without a source of nitrogen (nitrate or ammonium), to molybdenum and nitrogen deficient plants. Again the effects were revealed by the size and composition of the pool of soluble nitrogen compounds in leaves, and the recovery from the deficiency was followed with time.

(4) The effects of added molybdenum, combined with nitrate, on the size and composition of the pool of soluble nitrogen compounds in the roots of molybdenum deficient tomato plants.

The experiments here reported are only a part of the larger body of data which have been reported in full elsewhere (8). The details of experimentation to establish the desired levels of molybdenum deficiency and the visible symptoms produced have also been documented (loc. cit.), and they will not be recorded in detail here, since they are now somewhat familiar from such works as those summarized by Wallace (16) and Hewitt (6). The sampling and analytical procedures were comparable to those previously mentioned in the earlier papers. The alcohol soluble nitrogen compounds were extracted under conditions which preserved glutamine; the concentrated extract was analyzed by a procedure in which the various nitrogen compounds were separated by paper chromatography and then determined quantitatively with ninhydrin. The nitrogen in each such compound is expressed in micrograms of nitrogen per gram of fresh weight of tissue, and the total nitrogen in all the compounds so determined is taken as the size of the soluble pool of the compounds (amino acids and amides) which are related to protein metabolism. Amino acids which were detected, although present only in very small amounts (e.g., glycine and threonine), have been omitted from the tables. The experiments were necessarily carried out under the fluctuating conditions of a greenhouse. However, the effects due to the treatment are determined from a base which represents the composition of plants drawn from the same batch, but which were not affected by the variable, or variables, in question. The essential data are recorded in Tables I to IV, and the main conclusions to which they lead will now be presented.

COMPOSITION OF LEAVES OF DIFFERENT AGE IN RESPONSE TO FULL NUTRIENT SOLUTIONS CONTAINING NITRATE AND TO NUTRIENTS DEFICIENT IN MOLYBDENUM

The data which resulted from this experiment are shown in Table I. Terminal leaves were leaves at or above the third node behind the shoot apex; basal leaves were leaves below this node. It is not unexpected that the younger leaves would be richer in the soluble nitrogen compounds than the older ones, the former may in fact deplete the latter. It is also not unexpected that the younger, more actively growing, terminal leaves of full nutrient plants would be richer in glutamine and glutamic acid than leaves which are somewhat older. Moreover, in tomato, asparagine is often not present in leaves grown on nitrate as the source of nitrogen (14). Thus, the analysis in Table I of the leaves of plants grown on a full nutrient solution, which contained nitrate and molybdenum, presents no unexpected features. In comparison with this, however, the plants which had adjusted to lack of molybdenum, even to show visible symptoms, were surprisingly similar in the size and relative composition of the pool of soluble nitrogen compounds. While the disparity between the total soluble nitrogen of young and old leaves may be somewhat greater in the plants which lack molybdenum, the comparison of leaves in each age group, with and without molybdenum, produces little evidence that is outstanding, with the possible exception of the lower content of glutamine in the younger, molybdenum deficient leaves. This does not mean that molybdenum is without effect on the nitrogen metabolism of tomato leaves. It does mean, however, that molybdenum affects the nitrogen metabolism early in its course from nitrate, and then the subsequent course of nitrogen metabolism is not greatly affected qualitatively by the presence or absence of molybdenum. The pool of soluble nitrogen compounds must adjust to the total growth which these nutrient and environmental conditions can support. If lack of molybdenum exerted some specific effect on a particular and relatively late stage of nitrogen metabolism, one would expect to find more evidence of this in the accumulation, or otherwise, of some particular nitrogenous metabolite. After protracted growth under molybdenum deficient conditions, evidence of this is lacking. However, the observations made during the period of recovery from molybdenum or nitrogen deficiency do show the points at which molybdenum may intervene in the nitrogen metabolism by effects on the pool of given compounds which are seen before the restored processes of growth tend to reestablish anew a state of balance with the soluble compounds which are present.

TABLE I

DISTRIBUTION OF NITROGEN IN THE PREDOMINANT SOLUBLE NITROGEN COMPOUNDS IN BOTH OLDER AND YOUNGER LEAVES OF TOMATO PLANTS SUPPLIED WITH NITRATE-NITROGEN IN THE PRESENCE OR ABSENCE OF MOLYBDENUM (NITROGEN IN EACH COMPOUND EXPRESSED AS μG. PER G. FRESH WEIGHT)

Nitrogen compound detected	Amount of nitrogen in leaves from			
	Plants on complete nutrient solution		Plants deficient in molybdenum	
	Basal leaves	Terminal leaves	Basal leaves	Terminal leaves
Aspartic acid	18	19	10	22
Glutamic acid	29	39	23	35
Serine	14	16	10	36
Asparagine	0	0	0	0
Alanine	9	9	4	15
Glutamine	2	31	5	19
γ-Aminobutyric acid	16	15	11	33
Total nitrogen in ninhydrin reactive compounds	88	133	63	171

SIZE AND COMPOSITION OF THE POOL OF SOLUBLE NITROGEN COMPOUNDS IN TOMATO LEAVES DURING THEIR RECOVERY FROM MOLYBDENUM DEFICIENCY IN THE PRESENCE OF NITRATE

The data which are contained in Table II indicate the following:—

(1) When molybdenum is resupplied to tomato plants grown on

nitrate, but which had been rendered deficient in molybdenum, the plants can speedily use nitrate in excess of their immediate ability to consume the soluble compounds in protein synthesis and growth. This results in an increase in the total soluble nitrogen content, though this increase is temporary, for, as growth is resumed, the balance between the soluble nitrogen pool and its use in growth and synthesis tends to be re-established. These effects are even more conspicuous, as expected, in the younger leaves near the shoot apex, though they are also apparent in older leaves.

(2) Virtually all the principal constituents of the pool of soluble nitrogen compounds in leaves respond in the same manner as the total soluble nitrogen (except perhaps γ-aminobutyric acid), but the main response is in the amide nitrogen. Twenty-four hours after molybdenum was supplied, the total amide nitrogen of the younger leaves had increased approximately eightfold, and, following the earlier build-up of glutamine which was conspicuous at six hours, asparagine appeared temporarily in leaves in which it had not previously occurred in significant amounts. (In this respect, recovery from molybdenum is different from recovery from manganese.) Similar effects occurred in older leaves, although at the lower total content of nitrogen which is consistent with their stage of development.

TABLE II

DISTRIBUTION OF NITROGEN IN THE PREDOMINANT SOLUBLE AMINO COMPOUNDS IN THE LEAVES OF TOMATO PLANTS RECOVERING FROM MOLYBDENUM DEFICIENCY WHILE CONTINUOUSLY PROVIDED WITH NITRATE (NITROGEN IN EACH COMPOUND EXPRESSED AS μG. PER G. FRESH WEIGHT

Position of leaves sampled	Time after supplying Mo (hours)	Aspartic acid	Glutamic acid	Serine	Glycine	Asparagine	Alanine	Glutamine	γ-Aminobutyric acid	Total nitrogen in ninhydrin reactive compounds
Terminal	0	22	35	36	0	0	15	19	33	171
	6	70	83	53	56	0	41	137	16	503
	24	48	80	15	0	44	23	131	16	359
	30	16	37	6	7	19	4	44	7	146
Basal	0	10	23	10	0	0	4	5	11	63
	6	27	43	24	23	0	19	101	7	263
	24	16	37	6	0	24	9	28	8	127
	30	26	46	13	0	0	4	59	10	165

(3) These results are obviously consistent with the view that molybdenum deficiency had affected nitrogen metabolism by limiting the ability of the leaves to utilize nitrogen from nitrate and that when this ability is restored the synthetic apparatus converted the reduced products from nitrate into a variety of soluble compounds (amides and glutamine in particular) in excess of the immediate ability of the plant to utilize them in synthesis and growth.

It is to be noted here that Possingham (11) observed increases in the concentrations of amino acids when molybdenum was added to otherwise deficient tomato plants, but in short periods (2 hours) prior to this, actual decreases were noted.

CONTRASTED EFFECTS OF NITRATE AND AMMONIUM ON THE SIZE AND COMPOSITION OF THE POOL OF SOLUBLE NITROGEN COMPOUNDS IN PLANTS WHICH ARE RECOVERING FROM NITROGEN AND MOLYBDENUM DEFICIENCY

The general design of this experiment was similar to that recorded in Table II, except that the previously deficient plants were divided into two portions during the recovery period; half of them received molybdenum with and without nitrate, the other half received molybdenum with and without ammonium. The data are recorded in Table III.

The main features to be noted are:—

(1) The leaves of plants rendered both molybdenum and nitrogen deficient, and in which growth was severely limited by these deficiencies, contained soluble nitrogen at a low level and in the relative amounts designated in the table as at zero time. This is the base line from which the subsequent effects are to be measured.

(2) If the recovery from nitrogen deficiency (in presence of molybdenum) is from an ammonium source, there is a rapid and progressive increase of soluble nitrogen, and the amides (glutamine and asparagine) are the conspicuous components. This has been shown previously to be characteristic of leaves of tomato plants grown on ammonium and nitrogen and again, it is to be noted, asparagine appears in quantity in the plants which received ammonium, although it was absent, or scanty, in those which received nitrate, at least until a considerable level of soluble nitrogen was attained.

(3) If recovery from nitrogen deficiency was from an ammonium nitrogen source, this could occur almost equally well, both in the amount of soluble nitrogen accumulated and in the particular compounds accumulated, whether molybdenum was added or not. Hence, molybdenum deficiency must not have seriously impaired the enzymes which combine reduced forms of nitrogen with carbon acceptors of nitrogen to form the observed range of soluble compounds.

(4) The results of the experiment of Table III are essentially confirmatory of those shown in Table II insofar as the response of deficient plants to nitrate and/or molybdenum is concerned. While the response recorded in Table III was similar in kind, it was somewhat less striking in degree, due mainly to a less dramatic accumulation of glutamine in these plants in the presence of both nitrate and molybdenum. However, the trend of the observed results and the essential conclusions are the same from Table III as from Table II so far as nitrate and molybdenum are concerned.

TABLE III

EFFECT OF MOLYBDENUM UPON THE PREDOMINANT SOLUBLE AMINO COMPOUNDS IN THE LEAVES OF TOMATO PLANTS RECEIVING NITRATE OR AMMONIUM NITROGEN (NITROGEN IN EACH COMPOUND EXPRESSED AS μG. PER G. FRESH WEIGHT)

Nutrient solution		Time of harvest (hours)	Aspartic acid	Glutamic acid	Serine	Asparagine	Alanine	Glutamine	Lysine	Arginine	γ-Aminobutyric acid	Total nitrogen in ninhydrin reactive compounds
N source	Mo status											
Nitrogen deficient	Molybdenum deficient	0	0	7	10	0	7	11	0	13	32	101
Nitrate	Molybdenum deficient	2	4	6.5	10	0	11	9	20	15	28	136
		6	3	3	10	0	10	8	11	14	35	113
		24	0	8	14	0	14	9	15	17	36	138
		30	5	9	12	0	14	9	18	19	19	129
Nitrate	Molybdenum supplied	2	6	0	11	0	18	17	23	27	33	173
		6	9	0	10	0	17	31	13	17	32	155
		24	9	4	8	0	20	51	11	14	34	172
		30	23	0	14	12	26	90	15	25	36	274
Ammonia	Molybdenum deficient	2	4	8	13	14	11	15	0	19	25	141
		6	6	12	14	15	8	25	0	15	23	141
		24	7	5	20	21	13	58	15	12	37	230
		30	7	6	26	28	15	122	17	27	40	329
Ammonia	Molybdenum supplied	2	3	8	12	12	9	21	16	20	19	146
		6	8	5	10	18	15	37	26	30	33	216
		24	8	4	12	26	18	51	18	23	34	229
		30	7	0	11	22	15	108	11	20	36	259

EFFECTS OF MOLYBDENUM ON THE SIZE AND COMPOSITION OF THE POOL OF SOLUBLE NITROGEN COMPOUNDS IN TOMATO ROOTS SUPPLIED WITH NITRATE

The nitrogenous composition of tomato roots from plants grown in the presence of nitrate, but deficient in molybdenum, is shown at zero time in Table IV. As previously noted, asparagine and arginine are conspicuous in the soluble nitrogen of roots, though not in leaves. This analysis is not greatly dissimilar in any way that can be specified from the relative composition of the pool of soluble nitrogen in roots of full nutrient tomato plants which had this general content of soluble nitrogen (9; cf. Table V, p. 433). However, when molybdenum is restored, the roots can accumulate soluble nitrogen from nitrate in excess, at least temporarily, of the ability of the plant to use it in growth and protein synthesis. The compounds principally responsible for this temporary build-up of soluble nitrogen in roots are, however, glutamine, asparagine, and glutamic acid.

TABLE IV

Distribution of Nitrogen in the Soluble Amino Compounds in the Roots of Tomato Plants Supplied with Nitrate during the Period after Correction of Molybdenum Deficiency (Nitrogen in Each Compound Expressed as μG. per G. Fresh Weight)

Hours after receiving Mo	Aspartic acid	Glutamic acid	Serine	Asparagine	Alanine	Glutamine	Arginine	γ-Aminobutyric acid	Total nitrogen in ninhydrin reactive compounds
0	9	29	5	17	4	9	16	12	107
6	12	52	2	59	4	14	9	6	166
24	8	38	0	18	6	44	18	8	151
30	8	42	1	17	4	18	16	5	125

CONCLUSIONS

The main effects attributable to molybdenum so far as these can be discerned from the analysis of the soluble nitrogen compounds have already been noted. It is only necessary to draw attention, in conclusion, to some main points and to make some comparisons with the similar experiments with manganese which are separately reported (14).

Molybdenum has demonstrable effects upon the soluble nitrogen compounds which occur free in tomato leaves and roots. These effects are apparent when deficient plants recover in the presence of both molybdenum and nitrate, but not when they recover in the presence of nitrate alone. Recovery from nitrogen deficiency is unaffected by molybdenum if the source of nitrogen is ammonium. Apparently the ability of the tissue to accumulate high levels of soluble nitrogen (particularly the amides, glutamine in leaves, asparagine in roots) does not involve molybdenum specifically. All these observations are consistent if the primary effects of molybdenum are exerted through its now well known role in nitrate reduction. However, the accumulation of soluble products from nitrate when molybdenum is restored is transient, and this implies that the nitrogen metabolism re-establishes a balance with the whole course of normal growth and protein metabolism which may be renewed on the addition of molybdenum. Whether molybdenum plays any other part than that here attributable to its role in nitrate reduction will need to be examined in a later paper. In a recent review of the relationship between molybdenum and nitrogen metabolism (to which the authors have had prior access), Hewitt (6) distinguishes two types of response. When plants are grown with nitrate, the main response to lack of molybdenum is to be seen in the decreased concentration of many amino acids. This is the expected response to molybdenum that flows from its role in nitrate reductase. However, under circumstances in which nitrogen metabolism is not being limited by nitrate reduction, molybdenum deficiency may cause the concentrations of certain amino acids to increase (e.g. arginine, γ-aminobutyric acid, serine), and Hewitt sees evidence of this effect in some of Possingham's data on tomato.

The outstanding differences between the responses attributable to molybdenum and to manganese, as seen in the soluble compounds of the tomato, are as follows. The responses due to molybdenum are clear-cut and direct, and they are exerted early in the course of the nitrogen metabolism as it stems from nitrate. Any repercussions this may have indirectly on the subsequent course of nitrogen metabolism, growth and protein synthesis seem to be attributable to the quantity of metabolism that can occur from nitrate, and not to any qualitative differences in the reactions which proceed from the first soluble organic nitrogen compounds which accumulate. In this respect, manganese and molybdenum contrast sharply.

Manganese exerts its undoubted effects on nitrogen metabolism, not upon nitrate reduction, but on the utilization of the reduced forms of nitrogen largely through the supply of carbon acceptors via the Krebs cycle and the course of carbohydrate metabolism. The effects of manganese seem also to extend to the subsequent course of protein synthesis through effects which regulate the balance between free (soluble) and combined (insoluble) forms of nitrogen. In this respect, therefore, the effects attributable to manganese are more varied, less direct, more diffuse, and they clearly interact to a much greater extent with the environmental and the developmental conditions which affect the behavior of the tomato plant.

By contrast, the effects due to molybdenum are much more direct than those of manganese and are localized in the immediate utilization of nitrate by reduction; molybdenum seems not to affect, qualitatively, the subsequent course of metabolism, though it may, by affecting growth, affect its rate.

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INTERNAL STANDARDS IN THE ANALYSIS OF CHLORINE-CONTAINING HERBICIDES BY GAS CHROMATOGRAPHY

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SUMMARY

The methyl esters of various halogenated phenoxyacetic, benzoic, and terephthalic acids can be separated by gas-liquid chromatography on silicone columns at temperatures ranging from 180° to 212° C. Herbicide residues in foods can be determined without quantitative handling of the sample by adding an internal standard prior to extraction. The standard should have physicochemical characteristics similar to those of the pesticide except for retention volume. Thus, 2-chloro-4-bromophenoxyacetic acid is a suitable standard for the analysis of 2,4-dichlorophenoxyacetic acid. This technique is similar in many respects to isotope dilution.

INTRODUCTION

Some herbicides containing both aromatic rings and carboxyl groups, as exemplified by derivatives of benzoic, phenoxyacetic, and terephthalic acids can be analyzed by conventional colorimetric methods. Thus, residues of 2,4-dichlorophenoxyacetic acid can be measured by the amount of formaldehyde evolved on treatment with sulfuric acid (8), or the amount of 2,4-dichlorophenol formed on splitting the ether linkage with pyridine hydrochloride (9). The dimethyl ester of tetrachloroterephthalic acid can be analyzed directly by measuring the intensity of the color produced on causing it to react with α-(4-nitrobenzyl)pyridine (10). However, until the advent of some of these recent developments (9, 10), methods for these compounds were less satisfactory than for any other prominent group of pesticides.

One reason for this is that, except for dimethyl tetrachloroterephthalate, these chemicals do not contain highly reactive functional groups that can be converted to chromophores without fundamental chemical modification of the molecules. However, the methyl esters of the phenoxyacetic and benzoic acid derivatives as well as dimethyl tetrachloroterephthalate are moderately volatile and, therefore, are amenable to gas chromatography. Thus, the potential exists for a simple general method which is applicable to all of these compounds.

In this work, various members of these series were separated by gas chromatography with the object of finding compounds closely related to commercial pesticides which are suitable for use as internal standards. By

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this technique, losses inherent in extraction, partial purification, and esterification can be minimized.

MATERIALS AND METHODS

Chemicals

The 4-chlorophenoxyacetic acid, 4-bromophenoxyacetic acid, 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and hexachlorobenzene were obtained from Eastman Organic Chemicals, Rochester, N. Y., and the BF_3-methanol reagent was obtained from Applied Science Laboratories, Inc., University Park, Pa. The dimethyltetrachloroterephthalate and related compounds and the benzoic acid derivatives were obtained from the Diamond Alkali Company, Painesville, Ohio.

Apparatus

The chromatograph was a Model G-100 Chromatograph manufactured by the Dohrmann Instrument Co., Palo Alto, Calif. and equipped with a microcoulometric titration cell. The chromatographic column used for all of the work was a 6 foot by $\frac{1}{4}$ inch column packed with 20 per cent (w/w) Dow Corning High Vacuum Silicone Grease on acid-washed Chromosorb. Nitrogen was used as carrier gas at 20 p.s.i.g. inlet pressure and atmospheric pressure at the outlet. Oxygen was used in the combustion tube.

Synthesis of Bromopentachlorobenzene[3]

Pentachloroaniline (15 g.) was suspended in 200 ml. of cold (−5° C.) concentrated H_2SO_4 in a precooled mortar. Powdered sodium nitrite (15 g.) was added slowly while stirring with a pestle and keeping the temperature below 5° C. The mortar and its contents were placed in a precooled container together with soda-lime to absorb fumes, and were kept at 5° C. for 20 to 30 hours. The reaction mixture was carefully poured onto about 600 g. of crushed ice with stirring. Then 30 g. of sulfamic acid was added to react with the excess nitrite in the cold solution. A small amount of scum plus any unreacted amine was removed by filtering the cold solution through a sintered glass Buchner funnel. The clear filtrate was added to a solution made by dissolving 20 g. of cuprous oxide in 200 ml. of 48 per cent HBr and heating on a steam bath until the evolution of gas was completed. After cooling, the solids were collected on a sintered glass Buchner funnel and washed with concentrated HCl and then with water to remove copper compounds. The residue was taken up in benzene, recrystallized from 2 liters of 2-propanol, filtered, and evaporated to dryness. It was then recrystallized from cyclohexane to give needle-like crystals melting at 233° C. (uncorrected).

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[3] The authors wish to thank Dr. E. A. Prill for the preparation of bromopentachlorobenzene, 2-chloro-4-bromophenoxyacetic acid, and the esters of the benzoic acid derivatives.

Prior attempts to make this compound according to the method in the literature (1, p. 210) by heating bromobenzene with anhydrous ferric chloride gave only hexachlorobenzene, possibly as a result of heating too drastically.

Synthesis of 2-Chloro-4-bromophenoxyacetic Acid

4-Bromophenoxyacetic acid was chlorinated with sodium hypochlorite by the general method of Hopkins and Chisholm (7). The product was recrystallized from cyclohexane, and the melting point was found to be 144° C. (uncorrected).

Preparation of Esters of 2,4-Dichlorophenoxyacetic Acid and Related Compounds

With HCl-methanol. Fifty to 100 ml. of 5 per cent HCl in dry methanol was added to 1 g. of the free acid in a round-bottomed flask and refluxed for 2 hours. Care was taken to keep the system free of water to obtain the methyl ester. The methanol was heated slightly and evaporated using a gentle stream of clean dry air. The residue was quenched with water and extracted with two 50-ml. portions of ether. The ether extract was washed with a dilute aqueous solution of $NaHCO_3$ and dried over magnesium sulfate. The ether was evaporated and the methyl ester of the acid remained.

With BF_3-methanol. Fifteen to 20 ml. of BF_3-methanol reagent was added to 1 g. of the free acid and the solution was boiled on a steam bath for 4 minutes. The solution was quenched with water and extracted with two 25-ml. portions of ether in a separatory funnel. The ether extract was washed with dilute aqueous $NaHCO_3$ and dried over magnesium sulfate. The ether was evaporated and the methyl ester of the acid remained.

Preparation of Esters of Benzoic Acid Derivatives

The acid was heated with excess thionyl chloride in benzene for 8 hours. Then the solvent and excess thionyl chloride were removed by distillation under reduced pressure. An excess of absolute methanol was added and the solution refluxed for 2 hours. Then it was quenched with water and extracted with ether. The ether extract was washed with dilute aqueous $NaHCO_3$ and dried over magnesium sulfate. The ether was evaporated and the methyl ester of the acid remained.

PREPARATION OF ESTERS

Many of the benzoic acid derivatives and phenoxyacetic acid derivatives used as herbicides are free acids, and efforts to chromatograph them as such were not successful. Therefore, they were first converted to methyl esters and chromatographed as such.

Dimethyl tetrachloroterephthalate and related compounds are esters, so there was no need to modify them chemically prior to chromatography.

Efforts to chromatograph 2,4-D and related compounds without esterification were only partially successful, but the methyl esters could be resolved very well at 201° C. on a 6 foot chromatographic column packed with 20 per cent (w/w) Dow Corning High Vacuum Silicone Grease on acid-washed Chromosorb.

These compounds were not quantitatively methylated on a microscale by either method which was used. Treatment with HCl-methanol and BF_3-methanol, procedures commonly used to esterify fatty acids, was investigated (4). Over-all recoveries of known amounts of acids which were methylated and then injected into the chromatograph varied from 30 to 60 per cent. Diazomethane has been reported to give quantitative yields of 2,4-D methyl esters when used to esterify the free acid (11). However, because of its extreme toxicity this material was avoided in the development of a method for residue analysis for general laboratory use. Also, both HCl-methanol and BF_3-methanol were found to interesterify higher 2,4-D esters with the same percentage recovery as with the free acids. If any of these higher esters were present, therefore, they would be converted to the methyl esters at this step and would be chromatographed as such.

The derivatives of benzoic acid could not be methylated by the methods described above since many of them are sterically hindered by the presence of chlorine atoms in the 2- or 6-positions of the benzene ring. Therefore, a direct procedure applicable to all members of this series could not be used. Consequently, the benzoic acid derivatives were first converted to acid chlorides by treatment with thionyl chloride, and the acid chlorides then were refluxed with methanol to obtain the esters.

Only methyl esters were used in this investigation. Undoubtedly, it would be possible to convert the acids to higher esters and chromatograph them under these or similar conditions. They would be expected to have larger retention volumes in proportion to the number of carbon atoms in the alcohol. This property could be used to shift the chromatographic peaks arising from herbicides having carboxyl groups in case of coincidence with those of other pesticides. Also, it would be possible to shift the peaks to regions of low background in cases where it is necessary to use a nonselective detector. In this work, a detector sensitive only to halogen was employed (microcoulometer), so this expedient was unnecessary.

EFFECT OF SUBSTITUENTS ON RETENTION VOLUMES

The chlorobenzoic acids are usually marketed as complex mixtures, so it was of interest to measure the relative retentions of various members of this series containing 3 to 5 chlorine atoms (Table I). Fair resolution of a synthetic mixture of methyl esters was obtained on a silicone column at 180° C. (Fig. 1). However, the earlier peaks were somewhat crowded, while the last peak, arising from methyl pentachlorobenzoate, was broad, owing

TABLE I

INFLUENCE OF SUBSTITUENTS ON RELATIVE RETENTIONS OF METHYL BENZOATES. COLUMN: 6 FT. 20 PER CENT (W/W) DOW CORNING HIGH VACUUM SILICONE GREASE ON ACID-WASHED CHROMOSORB. TEMP., 180° C.; CARRIER GAS, N_2; INLET PRESSURE, 20 P.S.I.G.; OUTLET PRESSURE, ATMOSPHERIC

Substituent	Relative retention
2,4,6-Trichloro	1.00
2,3,6-Trichloro	1.19
2,4,5-Trichloro	1.30
2,3,4-Trichloro	1.50
2,3,5,6-Tetrachloro	2.05
2,3,4,5-Tetrachloro	2.60
Pentachloro	3.75

to longitudinal diffusion in the column. Better presentation of data was obtained by programming the temperature (Fig. 2). The chromatograph oven was maintained at 170° C. until the elution of all the trichloro compounds, then the temperature was increased at the maximum rate for the elution of the remaining derivatives.

As anticipated, retention volumes increased with the number of chlorine atoms in the benzene ring. There were also differences between isomers. Thus, all of the trichlorobenzoates were resolved or partly resolved from

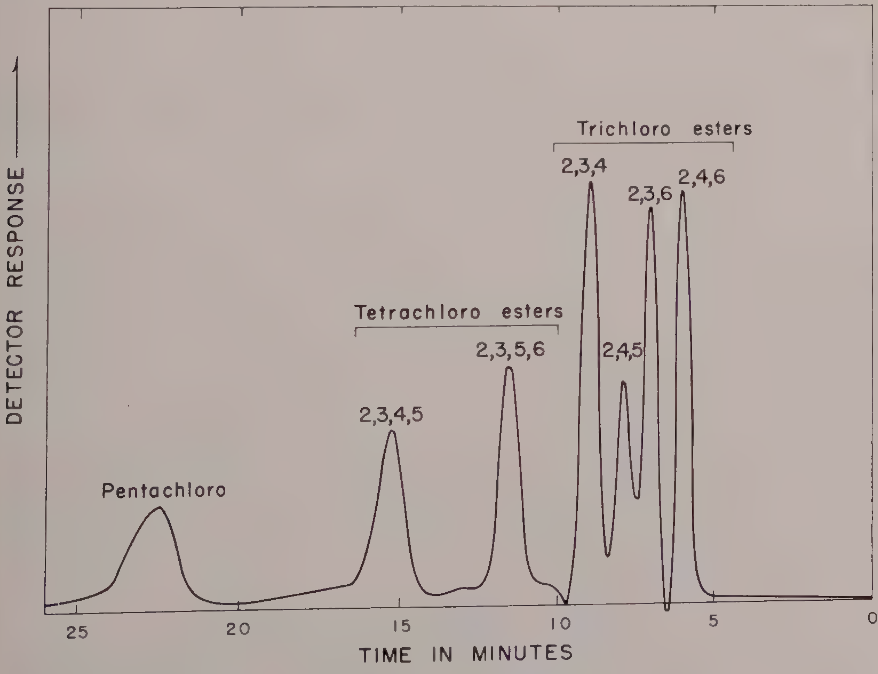

FIGURE 1. Separation of equal weights of methyl chlorobenzoates at 180° C.

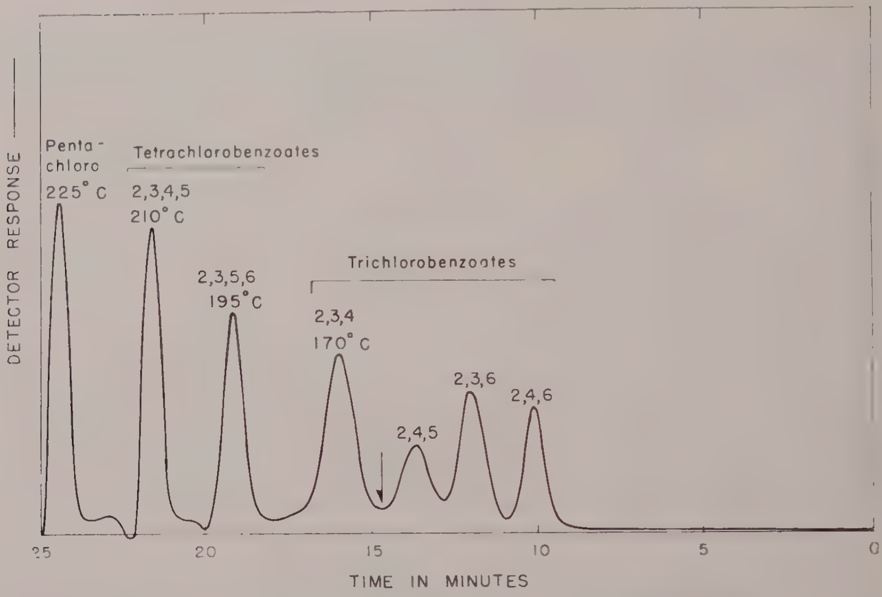

FIGURE 2. Separation of equal weights of methyl chlorobenzoates with the temperature programmed.

one another. All of the trichlorobenzoates are eluted ahead of the tetrachloro compounds. The 2,3,5,6-tetrachlorobenzoate was eluted ahead of the 2,3,4,5-isomer, showing that bunching of the halogens retards passage through the column.

The behavior of dimethyl tetrachloroterephthalate and related compounds is similar. On chromatographing a synthetic mixture of these materials on a silicone column at 212° C., it was found that retention volumes increased with the number of halogen atoms in the benzene ring (Fig. 3). Retention volumes were increased also by substitution of bromine for chlorine or by increasing the molecular weight of one of the alkyl groups. This former observation, although expected, suggests that bromine analogues of commercial pesticides might be good internal standards for use in gas chromatography. Of course, the example given here would not be useful, since the retention volumes of dimethyl tetrachloroterephthalate and methyl 2,5-dibromo-3,6-dichloroterephthalate differ too greatly. However, it is possible to obtain a more suitable standard by replacing only one of the chlorines with bromine.

SELECTION OF INTERNAL STANDARDS

Failure to obtain quantitative yields in the esterification of micro amounts of 2,4-D, and inadequacies in extraction procedures for milk

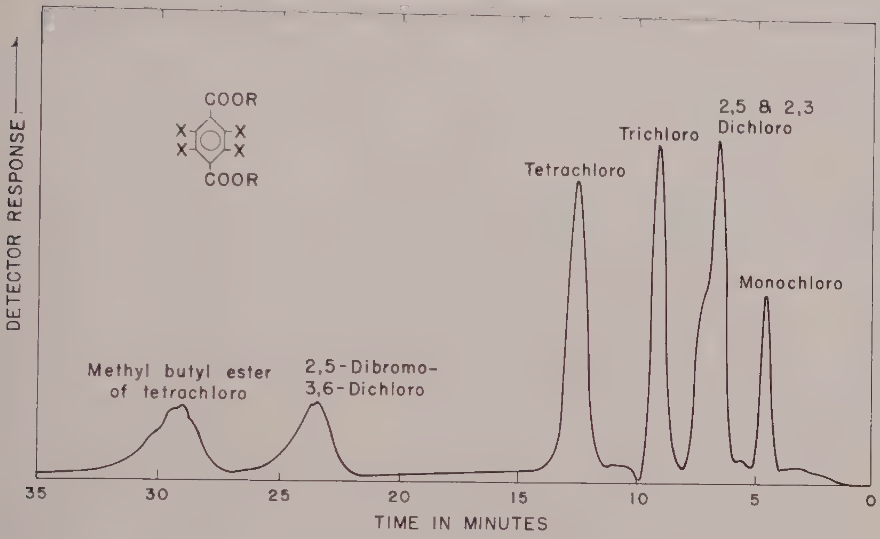

Figure 3. Separation of equal weights of dimethyl tetrachloroterephthalate and related compounds at 212° C.

samples[4] led to a consideration of the use of internal standards as a means of attaining more accurate analytical results. The use of internal standards is not new to gas chromatography. They are often employed when it is inconvenient to measure the amount of material injected into the chromatograph (4). A compound is selected having a retention volume close to that of the compound being analyzed. Solutions containing a constant concentration of standard and various concentrations of sample are prepared and chromatographed. The ratio of the peak area of the standard to that of the compound being analyzed is computed and plotted against the concentration of compound. Unknowns are treated in the same way, and the amount of compound present interpolated from the graph. The ratio of peak areas is essentially independent of concentration, so it is unnecessary to know the absolute amount of sample introduced into the chromatograph.

In most analyses, the internal standard is introduced at the last step only as a means of eliminating the necessity for accurate sample introduction. Therefore, the properties of the compound that is used are unimportant except that the retention volume must be close to that of the compound being analyzed.

However, it is believed that many of the difficulties attendant in residue analysis could be reduced or eliminated by adding a known amount of standard to the product being analyzed before the beginning of the analysis. Ideally, the standard should have physicochemical properties identical to those of the pesticide except for a difference in retention volume great enough to permit good peak resolution. The amount of pesticide in the food product then could be calculated from

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[4] Unpublished data.

$$p = (A/A_0)p_0R \tag{1}$$

where p is the p.p.m. of pesticide, p_0 the p.p.m. of standard, A the peak area of the pesticide, A_0 the peak area of the standard, and R an empirical correction factor determined by adding a fixed amount of standard and various known amounts of pesticide to the crop before making the analysis. When A_0/p_0A is plotted against p, the slope of the line will give the constant R. It may vary somewhat from crop to crop for each pesticide-standard combination. If R were unity, the method would be equivalent in principle to isotope dilution.

The following criteria are suggested in selecting a standard: 1) the molecular structure and physicochemical properties of the standard should approach those of the pesticide as closely as possible; 2) the retention volumes of the two compounds should be close but different enough to allow baseline resolution; 3) the experimentally determined value R should be as close to unity as possible; 4) the standard should not be a compound that is likely to be found as an impurity in the pesticide; and 5) the standard should not be another pesticide or have the same retention volume as known pesticides that might be carried through the analysis with it.

The most obvious choice would be to select a compound with one more or one less chlorine atom than the pesticide. Thus, dimethyl trichloroterephthalate could be used as an internal standard in the analysis of dimethyl tetrachloroterephthalate, since it emerges from the chromatographic column just before it (Fig. 3) and would be expected to have somewhat similar physicochemical properties with regard to solubility, volatility, partition coefficients, etc. However, it could conceivably occur as an impurity in this latter compound, which would violate requirement 4). Similarly, either 4-chlorophenoxyacetic acid or 2,4,5-T could be used as a standard in the analysis of 2,4-D since the methyl esters of the three compounds are well resolved when chromatographed on silicone at 201° C. (Fig. 4). However, 2,4-D and 2,4,5-T are often used in the same formulation, thus violating requirement 5). 4-Chlorophenoxyacetic acid is used in agriculture to a lesser extent, and might be acceptable in many applications.

Another procedure that would satisfy requirement 1) more completely would be to use a compound with the same number of halogens as the pesticide but in different positions. Thus, 2,3,4,5-tetrachlorobenzoic acid would be satisfactory to use in the analysis of 2,3,5,6-tetrachlorobenzoic acid, since the two compounds are well resolved chromatographically and

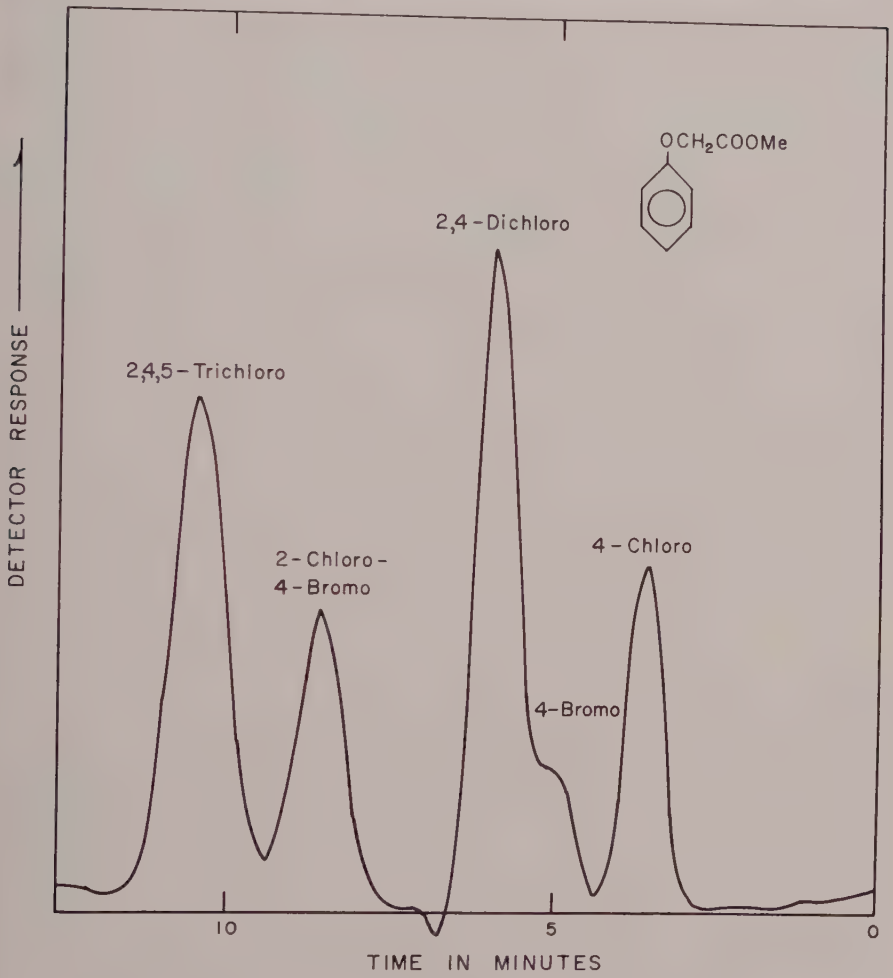


FIGURE 4. Separation of equal weights of methyl phenoxyacetates at 201° C.

would be expected to resemble each other more in physical properties than the pentachloro or any of the trichlorobenzoates. However, the two compounds might coexist in the same formulation, which would, of course, interfere with the analysis. Another example would be the use of 2,5-D as an internal standard in the analysis of 2,4-D. Unfortunately, the retention volumes of these compounds are too close to permit resolution with the column and operating parameters employed.

The most promising standards so far investigated have been obtained by replacing a single chlorine atom with a bromine atom. This will shift retention values far enough to permit good resolution even with polyhalogenated compounds. For example, bromopentachlorobenzene can be resolved cleanly from hexachlorobenzene, even though the two compounds resemble each other very closely in physicochemical properties (Fig. 5). Bromine substitution has several advantages for this application. These are: 1) retention volumes are shifted far enough to permit good resolution of pesticide and standard; 2) chromatographically speaking, one bromine atom is equal to about 1½ chlorine atoms. Thus, the peak obtained on the bromine analogue is likely to be in between that of the pesticide and the compound having one more chlorine atom, which would tend to minimize the possibility of errors introduced through the presence of impurities of this type; 3) the structural configuration of the molecule is identical to that

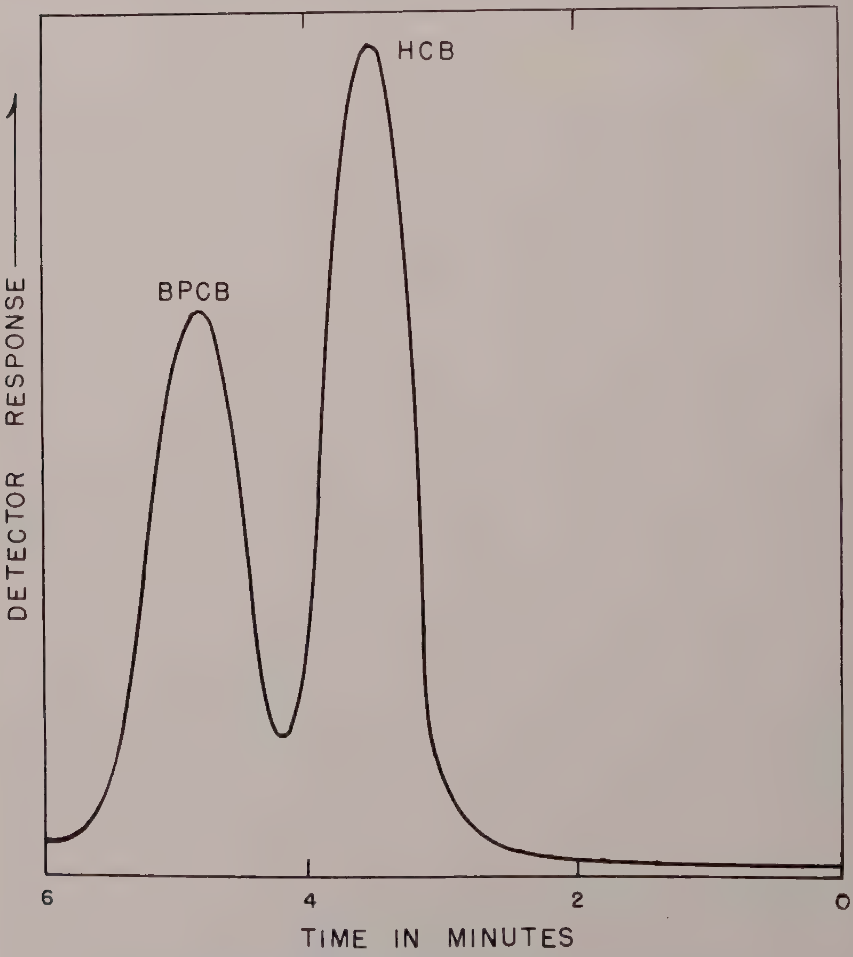


FIGURE 5. Separation of equivalent weights of hexachlorobenzene and bromopentachlorobenzene at 232° C.

of the pesticide with respect to the number and positions of halogen atoms, the only difference being in the size and electronegativity of one of them. This should result in very similar physicochemical properties, a conclusion which is borne out by the well-known fact that the biological activities of bromo compounds are often substantially equivalent to those of their chlorine analogues; and 4) compared to chlorine, bromine is quite expensive, so that it is unlikely to be used in agricultural applications where the chlorine analogue will work. Consequently, interferences caused by the presence of other pesticides are minimal.

The effect of bromine substitution on retention in the phenoxyacetic acid series is shown in Figure 4. 4-Chlorophenoxyacetic acid emerges from the column first, and is followed closely by the 4-bromo derivative. However, the peak arising from this latter compound overlaps the 2,4-D peak, so that it would not be a suitable standard under the operating parameters used in this experiment. Fortunately, the peak arising from 2-chloro-4-bromophenoxyacetic acid is intermediate between those of 2,4-D and 2,4,5-T. Improvements in column efficiency should permit baseline resolution. Therefore, 2-chloro-4-bromophenoxyacetic acid is a suitable internal standard for the analysis of 2,4-D with minimum interference from any 2,4,5-T that might be in the sample.

PROBLEMS IN DETECTION

The use of a detector specific for halogen is almost essential in analyses of this type. By employing alcohols of various molecular weights for esterification, it might be possible to prepare derivatives that would elute from the column in a region of low background. However, it is unlikely that a low enough background could be attained to permit use of an ionization detector.

Therefore, the best expedient is to combust the sample and measure the amount of chloride or chlorine produced. Chloride can be measured by collecting the combustion tube effluent in a suspension of mercuric chloranilate and measuring the amount of chloranilic acid liberated colorimetrically. This method is time-consuming and insufficiently sensitive for residue analysis at the microgram level. Another chemical method of analysis depends on the fact that chlorine or other oxidizing agents are produced when the oxygen content of the gas stream in the combustion tube is high. This oxidizing power can be measured by using a dilute solution of *o*-toluidine in aqueous HCl as the reagent. A brilliant yellow color is produced which can be measured optically. Inherently, the method is moderately sensitive, but the chlorine yield is poor (10 to 20 per cent of theory) and variable. Moreover, the platinum catalyst used in the combustion tube is easily poisoned, so sensitivity drops off rapidly with use.

The only detectors available commercially that will detect halogen selectively are the electron capture spectrometer (5) and the microcoulometer developed by Coulson *et al.* (6). Of these, the latter has been evaluated most thoroughly and was used in most of this work.

The microcoulometer is highly selective and sufficiently sensitive for residue analysis at the parts per billion level. It is essentially quantitative in response except when conditions are such that free halogens are formed in the combustion tube. Halogens will give only one-half of the response of an equivalent weight of halide ion, since on dissolving in the electrolyte they undergo the reaction

$$X_2 + H_2O \rightleftarrows H^+ + X^- + HOX \qquad (2)$$

The halide ion is titrated, but the hypohalite is not detected. Conditions which lead to halogen formation are the presence of an excess of oxygen in the gas stream (see above) and a deficiency of hydrogen in the compound being analyzed. Hexachlorobenzene, for example, contains no hydrogen whatsoever so therefore cannot form HCl on combustion. Consequently, the peak obtained on chromatographing it is considerably smaller than for pentachloroaniline, even though the latter compound contains less chlorine. This difficulty can be overcome by including a source of hydrogen in the gas stream. Passing the oxygen used in combustion through 0.5M ammonium hydroxide appears to be satisfactory.

Low results are also obtained when bromine is substituted for chlorine in the pesticide. This has been observed for bromopentachlorobenzene, dimethyl dibromodichloroterephthalate, methyl 4-bromophenoxy acetate, and methyl 2-chloro-4-bromophenoxy acetate. Detector response obtained on 2,4-D and related compounds is shown in Table II. The 4-bromo derivative gives only about 0.6 the response of the 4-chloro derivative indicating that most of the halogen is liberated as free bromine. 2,4-D gives about twice the response of the 4-chloro compound as anticipated. However, the 2-chloro-4-bromo derivative gives a response somewhat lower than the combined responses of the 4-chloro and 4-bromo compounds. Evidently, the two halogens interact in such a way that detector sensitivity is reduced.

Bromine is a less powerful oxidizing agent than chlorine and evidently

TABLE II

INFLUENCE OF SUBSTITUENTS ON MICROCOULOMETRIC DETECTOR RESPONSE TO METHYL PHENOXYACETATES

Substituent	Relative response
4-Cl	1.00
4-Br	0.61
2,5-DiCl	2.08
2,4-DiCl	1.98
2-Br-4-Cl	1.40

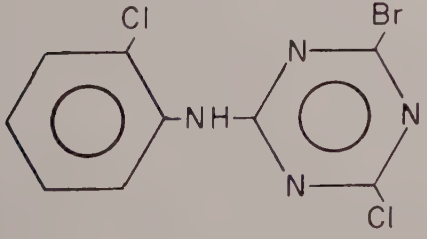
is liberated almost entirely as molecular bromine in the gas stream. As noted above, this reduces detector response by about one-half. It is probable that response to bromine could be improved by carrying out the combustion under reducing conditions. However, sensitivity is more than adequate, so there appears to be no compelling need for introducing this additional complication.

GENERAL DISCUSSION

The high resolving power of gas chromatography coupled with the use of a selective detector and a suitable internal standard by-passes many of the difficulties encountered in residue analysis. A number of compounds can be detected in a single run, sensitivity is vastly improved over colorimetric methods, and the need for quantitative extractions and transfers is greatly diminished. If an internal standard with ideal behavior were available, the only quantitative measurements that would have to be made would be the concentration of standard added to the sample and the ratio of peak areas. The need for quantitative extractions, complete breaking of emulsions, etc., would be eliminated, since sensitivity is high enough to withstand reasonable losses. However, in practice, the behavior of standards is not ideal, so extraction and partial purification must be carried out in a reproducible, although not necessarily quantitative, manner.

The best standards so far evaluated are obtained by substitution of a single chlorine atom with bromine. Peak resolution is good even for such closely related compounds as pentachlorobromobenzene and hexachlorobenzene. Detector response per bromine atom is only about one-half of that obtained with chlorine, but this is unimportant and probably could be corrected if necessary.

However, it should be emphasized that selection of a standard involves more than the arbitrary choice of a structural formula and its synthesis. For example, either compound I or II could be prepared for use as a standard for 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine, a commercially available fungicide.



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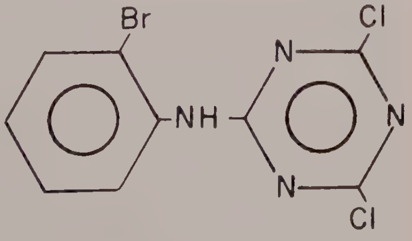
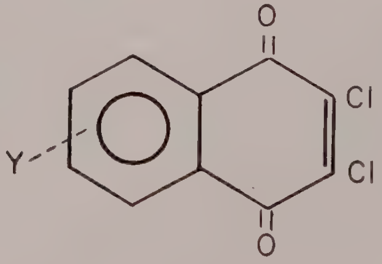


II

Structure I would be a poor choice, since kinetic studies have shown that the halogens attached to the heterocyclic ring are very labile and react rapidly with many metabolites present in food (2). Since bromine is more reactive than chlorine, compound I would disappear from the system much faster than the commercial fungicide, and therefore would be unsuitable as a standard. On the other hand, the halogen atom substituted in the benzene ring is very unreactive regardless of whether it is chlorine or bromine. Moreover, it has been shown by kinetic studies that the bromine atom in this position influences the chemical reactivity of the chlorine atoms in the heterocyclic ring in almost exactly the same way as chlorine. Thus, bromine substitution would be acceptable when judged by this criterion alone. However, it is known that there is considerable steric interaction between the two rings which is influenced by the size of the group in the *ortho* position (3). This has a pronounced effect on polarity and, hence, relative solubility, which in turn could influence factors important in isolation such as partition coefficients. Hence, it would be necessary to synthesize this compound and determine its recovery relative to 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine when extracted from various foods. Similar studies would have to be made on where to locate the bromine atom on DDT and other compounds.

In other cases, suitable standards could not be obtained at all by direct substitution of chlorine for bromine. For example, in the fungicide dichlone, the halogens are equivalent and therefore both are reactive.



Hence, the only practical alternative would be to substitute an additional group Y in the benzene ring that would increase the retention volume sufficiently to permit resolution from the fungicide. The choice would not be easy, since substitution of any group in the benzene ring is bound to influence the reactivity of the chlorine atoms to some degree.

There is an alternative which is not feasible at the present time but which, if realized, might greatly extend the capability of the gas chromatographic method. This would be to make a standard from heavy stable isotopes. The molecular weight of dichlone is 227, while the molecular weight of heavy dichlone (C^{13}, D, O^{18}, and Cl^{37}) is 248. The homologous series of saturated fatty acids differ in molecular weight by only 14, and it is possible to resolve these with great ease. Therefore, it should be possible to resolve dichlone and heavy dichlone, since the molecular weight difference is 21.

Although such a synthesis would be costly and difficult, it would be of great interest, since it would put gas chromatography directly in the isotope field. Such a method would be similar in principle to isotope dilution, but ease of sample handling and requirements for extraction would be vastly improved. A stock of heavy analogues of key pesticides, drugs, and metabolites would be of great value in checking the reliability of current analytical methods.

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BIOSYNTHESIS OF UNIFORMLY-LABELED SHIKIMIC AND QUINIC ACIDS IN LEAVES OF GINKGO BILOBA L.[1]

Leonard H. Weinstein, Clark A. Porter,
and Henry J. Laurencot, Jr.

SUMMARY

A simple and inexpensive method is described for the photosynthetic biosynthesis of uniformly-labeled shikimic and quinic acids using leaves of the maidenhair-tree (*Ginkgo biloba* L.). More than 2 grams of shikimic-U-C^{14} acid and about 0.5 gram of quinic-U-C^{14} acid were isolated. This procedure also allows for the isolation of other uniformly-labeled constituents such as sugars, amino acids, lipides, and nucleic acids which may be of further or future interest.

INTRODUCTION

Uniformly-labeled shikimic acid has been employed to demonstrate its role as an intermediate in the biosynthesis of a number of aromatic compounds in plants, among which are tyrosine and phenylalanine (3, 7), quercetin (13), cyanidin (4), and lignin (1, 2). Quinic acid has been shown to be readily converted by higher plants to shikimic acid and other aromatic compounds (15, 16, 17); therefore, it is also of interest in studies of aromatic biosynthesis.

The biosynthesis of shikimic-U-C^{14} acid by *Escherichia coli* 83-24 has been described (9, 11, 18). This is a very satisfactory method, but it has two major disadvantages. Firstly, optimal growth of *E. coli* 83-24 requires a concentration of 0.5 per cent glucose in the medium. This calls for either an enormous expenditure for uniformly-labeled glucose or dilution of the labeled glucose with unlabeled sugar. Since the specific activity of the product formed will be equal to that of the glucose supplied, it will result in shikimic acid of low specific activity. Secondly, the cost of uniformly-labeled glucose is relatively high when compared with $C^{14}O_2$, and only 4 to 5 per cent of the total carbon supplied from glucose is incorporated into shikimic acid. The bacterial method, however, has a major advantage; i.e., by the use of appropriately-labeled glucose, shikimic acid labeled in any desired position may be obtained.

The preparation of quinic-U-C^{14} acid biosynthesized photosynthetically from $C^{14}O_2$ by rose plants has been described (17). The yield of shikimic acid from this plant, however, was low.

A relatively simple and inexpensive procedure has therefore been devised by which large quantities of uniformly-labeled quinic and shikimic

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acids can be prepared photosynthetically. The subject for this biosynthetic procedure is leaves of the maidenhair-tree (*Ginkgo biloba* L.) which have been shown to contain a high level of both acids (5, 12).

MATERIALS AND METHODS

Biosyntheses were carried out at two different times of the year, in late winter and in spring.

Late winter biosynthesis. On February 7, 1962, several dormant branches from one-quarter to one-half inch thick were removed from a large *Ginkgo* tree at Boyce Thompson Institute. The branches were placed in water in a greenhouse. After 3 weeks the leaf buds had opened, and small leaves, about 5 to 10 mm. long (including the petiole), were evident. On February 28 woody spur branches, to which the leaves are attached, were removed from the branches. They were individually impaled on thumb tacks which had been inserted on three-quarter inch centers through a circular sheet of polyvinyl chloride, 23 cm. in diameter. A total of 105 spurs were accommodated. The plastic sheet and attached spurs were placed in a culture dish 23 cm. in diameter, 7.5 cm. deep, and containing about 1 cm. of distilled water. This was placed in turn into a 25-cm. diameter vacuum desiccator, having a total volume of 9.4 liters. The desiccator was placed in a greenhouse, and the leaves were supplied with additional light from high-intensity fluorescent lamps set for a 23-hour day. The desiccator was water cooled. The minimum temperature on the inside of the chamber was 15° C. at night, and the maximum was 30° C. during the day. Introduction of $C^{14}O_2$ was performed as shown in Figure 1. The apparatus shown was used for *Ginkgo* branches as described in the next section but was essentially the same as that used here.

The plant tissue was placed in water in the dish (B), inside of the chamber (A). Clamps (a), (b), and (d) were closed, and a vacuum was created in the chamber. Clamps (c) and (e) were then closed, leaving the chamber under vacuum. $C^{14}O_2$ was generated by passing 10 per cent perchloric acid through the funnel (E) into the tube (D) containing $NaHC^{14}O_3$. Before all of the acid had passed in, the stopcock on (E) was closed. Clamp (b) was opened first, followed by clamp (a). Ambient air was then drawn through the NaOH trap (c) which removed CO_2, swept through the $C^{14}O_2$ generation tube (D), and into the chamber (A). When the chamber (A) reached atmospheric pressure, clamp (b) was closed. The stopper at the top of the chamber was fitted with a thermometer (F), and a syringe (G), separated from the chamber by clamp (d). Aliquots of the gas in the chamber could be removed by opening clamp (d), and by pumping the plunger on the syringe (G) several times before removing an aliquot. The syringe was then quickly detached and fitted into a needle, previously inserted through a serum cap and dipped into NaOH or $Ba(OH)_2$ in a small centrifuge tube.

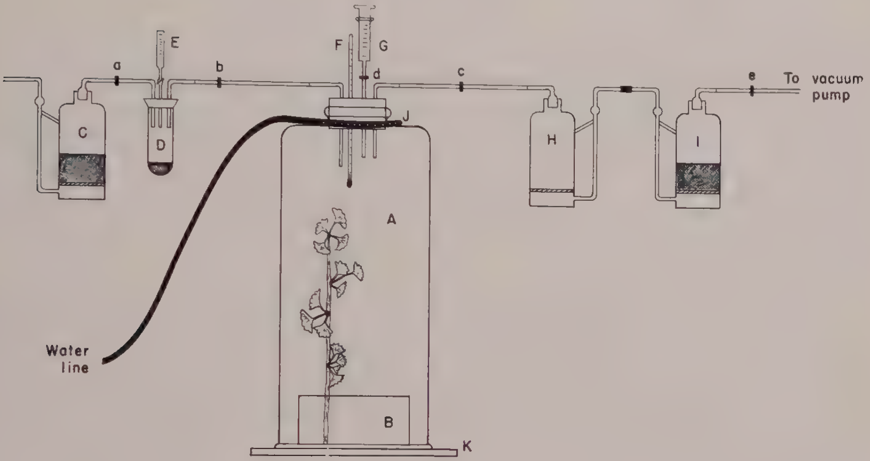

FIGURE 1. Apparatus for photosynthetic biosynthesis of uniformly-labeled shikimic and quinic acids. (A) Bell jar or desiccator; (B) crystallizing dish; (C), (I) NaOH traps; (D) $C^{14}O_2$ generating tube; (E) funnel for introduction of acid; (F) thermometer; (G) hypodermic syringe; (H) safety trap; (J) perforated rubber tubing; (K) glass plate; (a–e) screw clamps. (See text.)

$BaCO_3$ was then precipitated and counted. During bright sunny days the temperature was kept down in the chamber by running tap water slowly through a perforated rubber tubing (J). One millicurie of $C^{14}O_2$ was introduced into the chamber at 1- or 2-day intervals. This depended upon the amount of $C^{14}O_2$ remaining, and was determined by periodic removal of small aliquots of gas from the chamber. A total of 5 mc. of $C^{14}O_2$ was introduced over a 6-day period. After the last aliquot was introduced, the leaves were allowed to metabolize for 4 days. The chamber was then swept free of $C^{14}O_2$ by use of the vacuum pump attached to the NaOH trap (I) and the safety trap (H). Leaf tissues were then removed. Leaves had expanded to a length of 3 to 4 cm. Leaves and branch spurs were harvested separately, yielding a total of 20 grams of leaves and 30 grams of branch spurs.

Spring biosynthesis. On April 30, 30 *Ginkgo* branches each with leaves 3 to 5 cm. long were removed from trees and cut to 18 to 20 inches in length, bundled loosely with string, and placed in a 23-liter bell jar. The bases of the stems were immersed in distilled water contained in a culture dish. One millicurie of $C^{14}O_2$ was introduced into the chamber on April 30 and on May 4, while 2 mc. was passed in on May 7 for a total of 4 mc. The injections of $C^{14}O_2$ were not carried out on consecutive days because the light intensity from April 30 to May 5 was extremely low. Water was passed over the outside of the chamber for cooling. The temperature inside the bell jar ranged from a low of 20° C. to a high of 32° C. The branches were removed from the chamber on May 9. Only leaf tissues were harvested.

The leaves had expanded to a length of 7 to 8 cm. and weighed a total of 287 grams.

Extraction of tissues. The weighed tissues were immediately dropped into boiling 80 per cent ethanol and were ground in a VirTis "45" homogenizer. The homogenate was centrifuged and the supernatant liquid was decanted and saved. The residues were re-extracted four or more additional times with 80 per cent ethanol in a 50° C. water bath. Extracts were centrifuged and supernatant liquids were combined. The alcohol-insoluble residues were lyophilized.

Separation and isolation of quinic and shikimic acids. The ethanolic extract was passed through a bed of hydrogen-form Dowex 50-X4, 20 to 50 mesh, to remove free amino acids and amides. Five cubic centimeters of resin was used for each 20 grams of fresh weight of tissue. The resin was then washed with 80 per cent ethanol. The effluent liquid, which contained organic acids, sugars, and other neutral substances, was saved. If the uniformly-labeled free amino acids and amides are desired, they may be eluted as follows for each 5 cc. of Dowex 50: 40 ml. of 0.4N NH_4OH in 80 per cent ethanol, 15 ml. of 80 per cent ethanol, 15 ml. of water, 15 ml. of 4N aqueous NH_4OH, and 15 ml. of water (10). The combined eluates were dried and saved for future use.

The effluent fluid which passed through Dowex 50 was then taken up on acetate-form Dowex 1-X8, 200 to 400 mesh, bedded in ethanol. For 75 grams fresh weight of leaves a 2×30-cm. bed of resin was used. After the sample was passed through the column, the resin was washed with 80 per cent ethanol and then with 2 bed-volumes of water. The effluent liquid, which contained sugars and other nonionic substances, was saved and dried. The Dowex 1 was then eluted by a gradient of 2.5N acetic acid into water (6, 8, 16). The mixing flask contained 250 ml. of deionized water. A total of 150 5-ml. fractions was collected on a fraction collector at a flow rate of 1 ml. per minute. The fractions were then taken to dryness individually under a stream of air in a water bath at 40° to 45° C. (8). To each tube was added 1 ml. of water and 2 drops of indicator (0.2 per cent ethanolic bromcresol green -- 0.2 per cent methyl red, 5:1), and they were then titrated with 0.1N NaOH. A typical titration curve for about 75 grams of leaves is shown in Figure 2. The tubes representing each peak were combined and passed through hydrogen-form Dowex 50-X4, 20 to 50 mesh, to remove sodium and methyl red. The effluent, which contained the quinate or shikimate from 287 grams of leaves, was then taken up on a 2×20-cm. column of acetate-form Dowex 1-X8, 200 to 400 mesh, resin. The resin was washed with water and then with 100 ml. of 0.25N acetic acid. This step removed some yellow pigment but little or no quinate or shikimate. The resin was then eluted with 300 ml. of 2.5N acetic acid, which was then taken to dryness on a rotary vacuum evaporator. Shikimic acid dried to a white amorphous powder. Quinic acid dried to faintly yellow-colored crystals. The acids were not purified further.

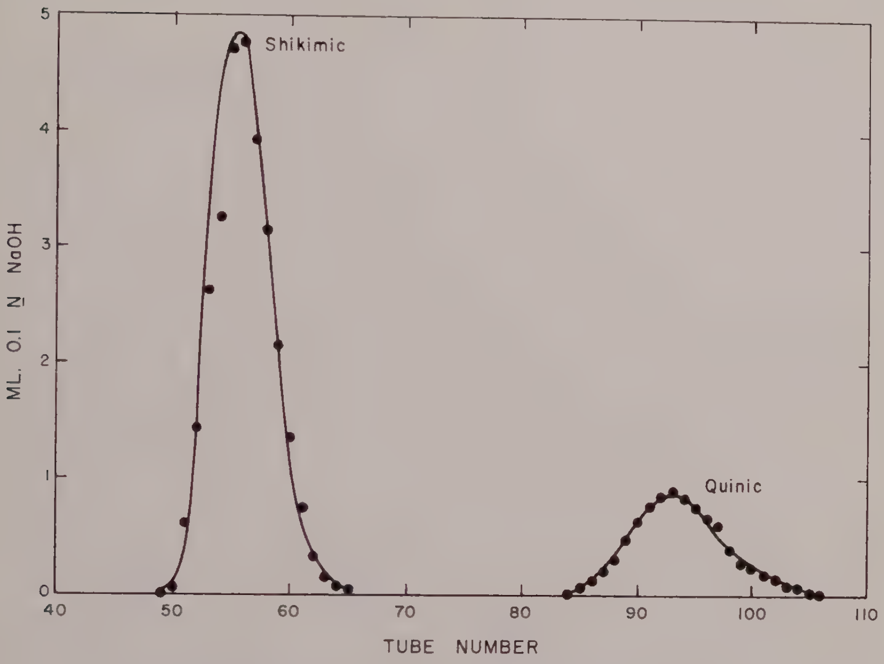

FIGURE 2. Gradient elution column chromatography of shikimic and quinic acids from *Ginkgo* leaves on acetate-form Dowex 1-X8, 200 to 400 mesh.

Isolation of sugars and lipides. If desired, mixed sugars may be isolated from the previously dried neutral fraction. The dry residue was taken up in 2 parts of water and 1 part of chloroform (or petroleum ether) and transferred to a centrifuge bottle. This was repeated several times until all of the residue was dissolved. The emulsion was centrifuged and the aqueous layer containing sugars was removed with a pipette. The organic phase was then washed with water by shaking, followed by centrifugation. The aqueous phase was removed and combined with the first. Both the aqueous and organic phases were taken to dryness, yielding crude mixed sugars and crude mixed lipides and pigments. These fractions may be purified by appropriate procedures such as column chromatography on cellulose powder for sugars and silica gel chromatography for lipides.

RESULTS AND DISCUSSION

The winter biosynthesis yielded 13 and 8 mg. of shikimic acid and 16 and 14 mg. of quinic acid from leaves and branch spurs, respectively. The specific activities (corrected for Geiger counter efficiency) were 4,550,000 c.p.m. for shikimic and quinic acids isolated from leaves and 173,000 c.p.m. per mg. for shikimic and quinic acids isolated from branch spurs (0.36 μc. and 0.014 μc./μM for shikimic acid and 0.40 μc. and 0.015 μc./μM for quinic acid).

The spring biosynthesis yielded 2.120 grams of shikimic acid and 0.593

gram of quinic acid. Specific activity was 191,000 c.p.m. per mg. (0.015 $\mu c./\mu M$ for shikimic acid and 0.017 $\mu c./\mu M$ for quinic acid). Infrared curves were identical with those for known samples, and melting points deviated no more than 4° C. from those reported.

Using the spring biosynthesis as a basis for calculations, the yield of shikimic acid was 0.74 per cent, and quinic acid was 0.21 per cent of the fresh weight of the harvested *Ginkgo* leaves. Shikimic acid occurred in a concentration 3.5 times that of quinic acid as compared to nearly equivalent concentrations in the very young leaves from the winter biosynthesis and from leaves harvested in late summer (12). Since 4 mc. of $C^{14}O_2$ was introduced into the growth chamber for photosynthetic fixation in the spring biosynthesis, and about 0.23 mc. was incorporated into shikimic and quinic acids, the biosynthetic efficiency was about 5.8 per cent. This value would have been unquestionably higher if the light conditions during biosynthesis had been more favorable.

In addition to the previously described advantages inherent in this procedure, i.e., use of relatively inexpensive $C^{14}O_2$ and the potential of producing large quantities of uniformly-labeled shikimic and quinic acids, certain other benefits accrue. The neutral fraction, after deionization with Dowex 50 and Dowex 1, partitioning with chloroform-water, and decolorization with charcoal, yielded 4 grams of uniformly-labeled sugars. A portion of this material, with no further purification, has already been used to produce anthranilic-U-C^{14} acid microbiologically. It contained 2.5 per cent raffinose, 47.8 per cent sucrose, 30.1 per cent glucose, and 19.5 per cent fructose as determined by paper chromatography (14). About 3 grams of nonvolatile organic acids was recovered by eluting the Dowex 1 columns with 6N formic acid after shikimic and quinic acids had been removed by gradient elution. More than 1 gram each of lipides and pigments and of free amino acids was also isolated. Twenty-eight grams of dry alcohol-insoluble residue was obtained, from which labeled amino acids and ribonucleic and deoxyribonucleic acids could be isolated.

This procedure could undoubtedly be used for the preparation of shikimic-U-C^{14} acid from other gymnosperms containing high levels of this compound.

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THE SULFUR METABOLISM OF INSECTS. VII. THE METABOLISM OF THE SULFUR AMINO ACIDS AND OF SULFATE IN THE BLOWFLY, PHORMIA REGINA (MEIG.)[1]

S. Mark Henry and Richard J. Block[2]

SUMMARY

The interrelationships of sulfate, cystine, and methionine in *Phormia regina* (Meig.) were investigated using S^{35}-labeled metabolites. The blowfly was unable to use sulfate for synthesis of the sulfur-containing amino acids. Methionine-S was converted to cystine-S and glutathione-S and was incorporated into protein. Cysteine and cystine were incorporated into protein and were degraded to taurine and sulfate but were not used in the synthesis of methionine. Sulfur metabolism in this insect, therefore, resembles that of most other insects and higher animals. Failure to synthesize methionine from cystine or sulfate is considered indicative of a dietary requirement for either methionine or a suitable thiomethyl precursor.

INTRODUCTION

A chemically defined diet for *Phormia regina* was described in 1956 by McGinnis, Newburgh, and Cheldelin (8). Among the amino acids listed as dispensable were cystine and methionine, but one or the other had to be present in the diet. One concludes that cystine and methionine are interconvertible in this animal. While methionine is well known to be metabolized to cystine in the metazoa, the reverse does not usually occur (1, 4). In those cases where cystine is converted to methionine, microorganisms are either suspected or known to be involved (2, 6). The present investigation was conducted in order to determine if *Phormia regina* can meet its methionine requirements from precursors other than thiomethyl compounds.

METHODS

Insects. The blowflies used in these experiments were obtained from Dr. V. J. Brookes of Oregon State College, Corvallis, Oregon. Adult flies were fed a 5 per cent w/v glucose solution except when oviposition was desired, in which case a small quantity of fresh beef liver was placed in the cage. Larvae were grown on liver in large, covered Petri dishes. A minimum of 24° C. was found to be desirable for oviposition.

Aseptic larvae were used for the cystine-S^{35} feeding experiment. The larvae were sterilized and cultured according to the procedures described by Cheldelin and Newburgh (3) except that either casein or lactalbumin was used in the basal diet. The use of lactalbumin, with a higher cystine-

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[1] Aided by grant E-2608, National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland.

[2] Deceased February 4, 1962.

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methionine ratio than casein, was expected to accelerate the conversion of cystine-S^{35} to methionine-S^{35}. One- by six-inch culture tubes, each containing 10 grams of diet, were inoculated with surface-sterilized blowfly eggs and were incubated at 25° C.

Isotopes and experimental procedures. Methionine-S^{35} was prepared as a 0.5 per cent w/v aqueous solution containing 0.16 microcurie per microliter. A five-microliter quantity of this solution was injected into each fly. Sodium sulfate-S^{35} was used as a carrier-free solution containing 1.2 microcuries per microliter. Each insect was injected with two microliters. The specific activities of the labeled cystine and cysteine[3] were not determined.

S^{35}-labeled cysteine, methionine, or sulfate was injected into either larvae or adult flies using a micrometer-controlled syringe fitted with a 30-gauge stainless steel needle. Cystine-S^{35} was incorporated into the diet used for aseptic rearing. At appropriate intervals following isotope injection or after six days of growth on the cystine-S^{35} medium, the insects were extracted and the proteinaceous residues were hydrolyzed as described in a previous publication (5).

RESULTS

Sulfate-S^{35} metabolism. Fifteen newly emerged adult blowflies, injected with a solution of $Na_2S^{35}O_4$, were separated into three groups of equal numbers which were homogenized after incubation periods of 20, 40, and 60 hours. Several unidentified S^{35}-labeled compounds were found on chromatograms of the 80 per cent ethanol-soluble fraction of these flies. Cystine-S^{35} and methionine-S^{35} were missing from the extract and the hydrolyzate. The S^{35} was almost completely excreted during the 60-hour incubation period.

Methionine-S^{35} metabolism. Methionine and methionine sulfoxide were the only radioactive metabolites found in either the 80 per cent ethanol-soluble fraction (Fig. 1 A) or the protein of blowflies two hours after injection with methionine-S^{35}. Two days after injection, practically all of the free methionine-S^{35} was metabolized. Taurine, glutathione, and several unidentified compounds were found in the free amino acid fraction (Fig. 1 B). The protein hydrolyzate (Fig. 2) contained methionine and cysteine.

Cysteine-S^{35} and cystine-S^{35} metabolism. Twenty-six hours after injection of cysteine-S^{35} into adult blowflies, taurine-S^{35} and barely detectable amounts of sulfate-S^{35} were found in the 80 per cent ethanol extract. Most of the S^{35}, however, was either eliminated (as sulfate-S^{35}) or incorporated into protein. An aliquot of the protein hydrolyzate contained labeled cystine (2,000 counts per minute) but no detectable methionine-S^{35}.

Cystine-S^{35} uptake by blowfly larvae on the synthetic diets was rather low. However, the protein hydrolyzates of these larvae contained easily detectable quantities of S^{35}, all of which was present as cystine or cysteine, even in the insects from diets containing lactalbumin.

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[3] Made by reduction of cystine-S^{35} with zinc in dilute hydrochloric acid.

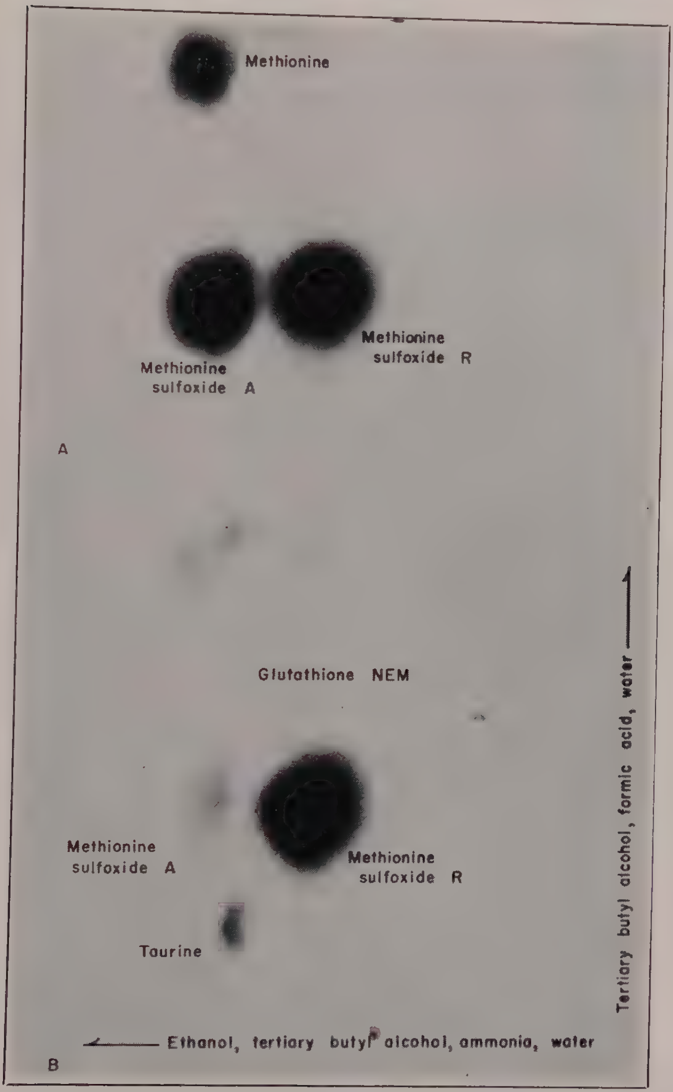

FIGURE 1. Autoradiogram of a chromatogram of an 80 per cent ethanol extract of *Phormia regina*. (A) Two hours after injection with methionine-S^{35}. (B) Forty-eight hours after injection. Two methionine sulfoxide spots are seen as a result of oxidation during chromatography. Methionine sulfoxide R may partially represent tissue sulfoxide. (NEM) *N*-ethylmaleimide.

DISCUSSION

The data presented herein are not nearly as complete as those obtained in studies on the housefly (4, 7) or the German cockroach (2, 6) but are sufficient to conclude that there are no major differences in sulfur metabolism in the two species of Diptera investigated. Neither species is able to synthesize methionine or cystine from sulfate; neither can convert cystine-S to methionine-S; both incorporate methionine and cystine into protein and degrade these amino acids to taurine and to sulfate. None of

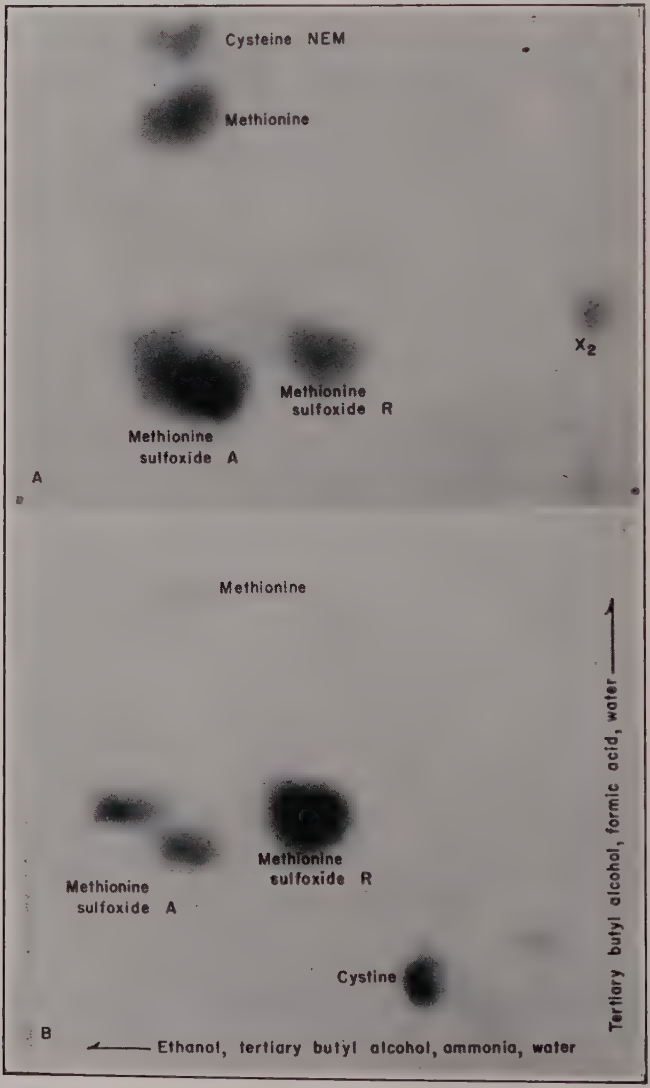

FIGURE 2. Autoradiogram of a chromatogram of an acid hydrolyzate of tissue remaining after extraction of *Phormia regina* injected with methionine-S^{35}. (A) Homogenized with *N*-ethylmaleimide. (B) Homogenized without *N*-ethylmaleimide. (X_2) Hydrolytic product of the *N*-ethylmaleimide adduct of cysteine. (See Fig. 1.)

this is peculiar to insects, for several other multicellular animals and some of the protozoa are known to metabolize sulfur compounds in a similar manner. Exceptions occur in ruminants, which are nutritionally dependent on internal, extracellular microorganisms and in such animals, as the cockroach, with active, intracellular microorganisms. Utilization of sulfate for amino acid synthesis and interconvertibility of cystine and methionine are also common in plants, bacteria, and fungi.

The data from the methionine-S^{35} injection experiments clearly show that oxidation of methionine to the sulfoxide occurs in the blowfly. About half of the free methionine is present in the oxidized form (designated as methionine sulfoxide R) on chromatograms of flies extracted two hours after injection. However, some of this is formed as the sample is drying after application to the chromatogram. Two days after injection (Fig. 1 B), methionine sulfoxide-S^{35} is the major radioactive spot on the chromatogram of the 80 per cent ethanol-soluble material. Methionine sulfoxide A on the chromatograms is entirely due to oxidation of methionine during drying of the chromatographic solvent after development in the first direction.

Ray and Koshland (9) report that methionine sulfoxide extensively reverts to methionine under the normal conditions of acid hydrolysis. It is believed, therefore, that most or all of the methionine sulfoxide on chromatograms of the protein hydrolyzates of *P. regina* is formed as a result of oxidation of methionine during preparation of the samples for chromatography.

The presence of cysteine (in the free form and as a constituent part of glutathione and protein) and almost complete absence of the oxidized form, cystine, are especially interesting. Cystine is found on chromatograms only when *N*-ethylmaleimide is not added to the homogenate (Fig. 2 B). The technique of preparing these extracts and protein hydrolyzates with and without *N*-ethylmaleimide not only indicates the normally occurring chemical form of the compound in question but also serves as a check on the identity of sulfhydryl compounds.

ACKNOWLEDGMENT

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SUMMARY OF PROCEDURES USED IN THE CONTROLLED FUMIGATION OF PLANTS WITH VOLATILE FLUORIDES AND IN THE DETERMINATION OF FLUORIDES IN AIR, WATER, AND PLANT TISSUES

R. Mavrodineanu,[1] J. Gwirtsman,[1] D. C. McCune,
and Clark A. Porter

Investigations at Boyce Thompson Institute on the effects of atmospheric fluorides on vegetation necessitated the development of procedures for the controlled fumigation of plants and for the analytical determination of fluorides in plant material, water, and air. These procedures were published as they were developed and modified over a period of several years. This paper summarizes the procedures currently employed and gathers in one place the references to the detailed technical papers necessary to reproduce them.

APPARATUS AND PROCEDURES USED IN THE CONTROLLED FUMIGATION OF PLANTS WITH VOLATILE FLUORIDES

The controlled fumigation of plants with volatile fluorides requires the delivery of a constant supply of material into the air stream entering the greenhouse or fumigation chamber. Methods and apparatus were developed and adapted to field use with portable greenhouses by which the concentration of hydrofluosilicic acid (H_2SiF_6) or hydrofluoric acid (HF) in the air stream was satisfactorily controlled (4, 5).

The fluoride dispensing apparatus is shown in Figure 1. The HF solution in flask B is atomized from a platinum nozzle by air regulated to a pressure of 15 to 20 pounds per square inch. The droplets (mean diameter about 10μ) are passed into a Teflon tube maintained at 150° C. where the acid is vaporized and introduced to the air stream entering the fumigation enclosure. The amount of hydrogen fluoride injected is proportional to the hydrogen fluoride concentration of the solution in flask B for a given nozzle and air pressure. The atomization characteristics of a nozzle are constant, and, if no clogging occurs and the air pressure remains constant, the apparatus will deliver the same amount of volatile fluoride for more than a month (5).

When H_2SiF_6 is atomized through a hot tube, it partially decomposes to silicon tetrafluoride (SiF_4) and HF. Thus, the fumigation is performed with a mixture of SiF_4, HF, and H_2SiF_6 in equilibrium according to the atmospheric temperature and humidity (5). HF solutions yield volatile fluoride

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in the form of HF if polyethylene equipment is used and no silica-containing material is present. Solutions of HF or H_2SiF_6 produce equivalent effects on various species of plants by the fumigation procedures described here if the concentrations of volatile fluoride in the fumigation chamber are equal (11). HF was used instead of H_2SiF_6 to avoid fumigating plants with a complex and qualitatively indefinite mixture of volatile fluoride.

A portion of the atomized solution condenses on the walls of flask C and returns to B and, owing to the evaporation of water, the hydrogen fluoride concentration of the returning solution is increased. The HF concentration of the solution in reservoir A is less than that of the solution in B to compensate for this loss of water. The correct dilution depends upon the characteristics of the individual apparatus and must be determined empirically. This laboratory has found that a solution one-third the concentration in B assured a constant supply of fluoride over a period of 7 days (5).

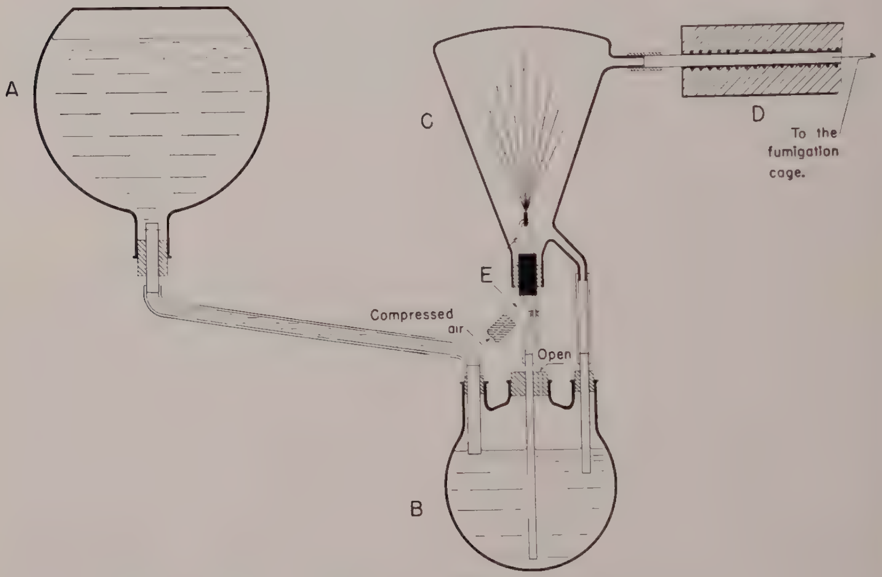

FIGURE 1. Unit for dispensing volatile fluoride. (Explanation in text.)

Beginning in 1961 a new method of fluoride injection was used. The methods and equipment described by Hill *et al.* (2) and McNulty and Newman (3) were modified and adapted for use with a new quonset type aluminum frame house covered with Type 20 clear Tedlar PVF film of 0.005-inch thickness (E. I. du Pont de Nemours & Co., Wilmington, Del.). In the new procedure the HF solution is not atomized, but a stream of air is passed through the solution and then into a Teflon hot tube. Over a two-week period the amount of HF lost from the solution was less than 5 per cent of the initial HF content. The control of the amount of fluoride injected is equal to that of the older method, and the reliability is greater owing to the elimination of the atomizer which was susceptible to clogging. The new equipment is more compact and the entire injection apparatus can be placed in a 15-gallon covered plastic container for use in field operations.

The heating tube was made from a 12½ inch length of ⅜ inch O. D. Teflon tubing into which threads had been cut over the complete length and was attached at a right angle to a Teflon rod extending into the plastic bottle containing the HF solution. This tube assembly was wound with slightly more than 20 feet of No. 26 Nichrome wire (Driver-Harris Co., Harrison, N. J.) having a resistance of 0.155 ohm per foot. The wire was wound into the threads cut into the tubing and exposed part of the rod. The tube and rod assembly was covered with a spiral wrap of ¾ inch wide asbestos tape, the ends of which were secured with a ring of plastic tape. A final wrap of Scotch No. 33 electrical tape (Minnesota Mining & Manufacturing Co., St. Paul, Minn.) completed the tube.

The power supply for the heating tube consisted of a filament transformer rated at 6.3 volts and 3 amperes. A 3-ohm 10-watt adjustable series resistor was used to limit the current to a value which would maintain the heating tube at a temperature of 147° F. This power supply is more economical than the Variac voltage regulator used by Hill *et al.* (2).

ANALYTICAL PROCEDURES

The method employed for the determination of fluoride has been adapted from the procedure of Willard and Winter (10). The specific requirements of the work at Boyce Thompson Institute necessitated modification of the basic procedure, improvement of equipment, and development of new techniques. The method used currently has evolved as the result of several thousand determinations, and its reproducibility has been evaluated in two cooperative projects involving 15 other laboratories (1).

Plant material. Plant material is dried in an oven at 70° to 80° C. to a moisture content of approximately 5 per cent. The sample is then powdered in a standard No. 3 Wiley cutting mill (Arthur H. Thomas, Philadelphia, Pa.) to give a preparation of 20 to 40 mesh. An aliquot of the sample is weighed in a tared nickel crucible, and fluorine-free calcium oxide is added (usually equal to 10 per cent of the weight). Moisture determinations can be made at the same time on 1 to 2 grams of the sample but are not made routinely. Fluorine-free distilled water is added to cover the material, the suspension is thoroughly mixed, and phenolphthalein is added to check the alkalinity.

A 1- to 5-gram aliquot of the sample is generally used for analysis, but the size of the aliquot may be adjusted according to the expected fluorine content. Aliquots of 0.1 to 10 grams from 20- to 25-gram samples of dried leaves which had been ground and thoroughly mixed gave comparable data on fluorine (1).

Fresh plant material may also be used for analysis. Fresh material is cut into fine pieces and placed in a tared Mason jar with fluorine-free calcium oxide (usually equal to 10 per cent of the sample dry weight), and the jar is closed tightly and reweighed. The moisture content of the material is determined at the time of sampling. The contents of the jar are transferred quantitatively to a 500- or 750-ml. tared porcelain casserole, covered with distilled water, and mixed thoroughly. Phenolphthalein is added to check the alkalinity of the suspension.

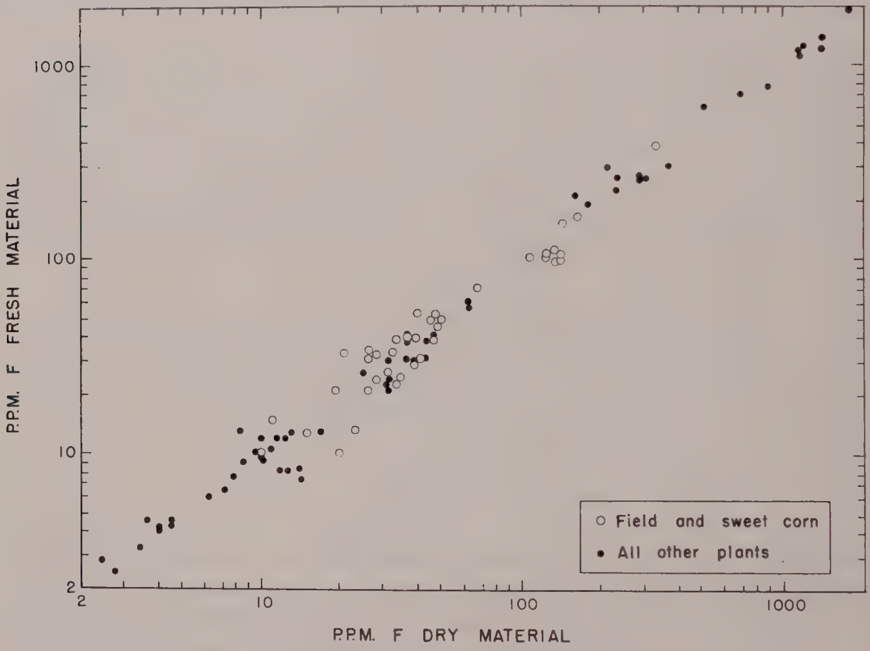

FIGURE 2. A comparison of the results of fluorine determinations on fresh and dried plant material (r = 0.989).

Comparison of the fluoride content of fresh vegetation fixed with calcium oxide and aliquots of the same material dried before fixing showed a high degree of correlation between the two procedures for many different crops. Data from 95 samples representing seven different crops are plotted in Figure 2 and show that the results obtained by the two procedures with comparable samples showed a correlation coefficient of 0.989. A routine procedure employing dried plant material for analysis has been preferred because of its convenience. However, the data in Table I show that anal-

TABLE I

EFFECT OF STORAGE AT ROOM TEMPERATURE ON FLUORINE CONTENT OF PLOT-GROWN ALFALFA HARVESTED MAY 29, 1956, AFTER A 6-DAY FUMIGATION WITH HF GAS. SAMPLES WERE COLLECTED IN TRIPLICATE, PLACED IN SEPARATE BAGS, AND ANALYZED AT THE FOUR SUBSEQUENT TIME PERIODS INDICATED BELOW. VALUES ARE PARTS PER MILLION OF FLUORINE

Time analyzed	Control		Average concentration HF, p.p.b.			
			8		17	
	Fresh*	Dry**	Fresh*	Dry**	Fresh*	Dry**
June 1956	18	9	203	168	282	272
	11	8	177	168	266	295
	9	10	206	176	314	263
Sample mean	13	9	195	171	287	277
July 1956	11	9	163	158	238	279
	13	9	163	160	271	267
	12	7	180	154	260	252
Sample mean	12	8	169	157	256	266
October 1956	14	9	203	180	289	250
	14	7	190	157	288	251
	10	7	232	163	288	264
Sample mean	13	8	208	167	288	255
January 1957	13	9	180	178	288	288
	11	10	190	210	295	293
	22	9	187	195	291	319
Sample mean	15	9	186	194	291	300
Mean	13	9	190	172	281	274
Least sign. diff., 5%	2.2		11		11	

* Fresh material was fixed with CaO at time of harvest and then stored.

** Dry material was air dried at time of harvest and then stored.

yses of alfalfa (*Medicago sativa* L.) fixed when fresh resulted in significantly higher fluoride values than did analyses of comparable dried samples when control (nonfumigated) or plants fumigated with 8 p.p.b. of HF were examined. Fresh and dried materials yielded equivalent results when the alfalfa plants were fumigated with 17 p.p.b. of HF. The possible effects of dew, rain, or dust were not considered in these experiments.

Ashing. The sample is placed on a hot plate under an infrared lamp to evaporate the water and char the plant material and then placed in an electric muffle furnace at 600° C. for approximately 2 hours.

Fusion. Sodium hydroxide pellets are added to the ash (5 grams of sodium hydroxide to 1 gram of ash), and the contents of the crucible are fused at 600° C. for 10 minutes in the electric furnace. If the total ash exceeds 1 gram in the analysis of fresh plant material, a 1-gram sample should be taken for fusion.

The presence of excessive amounts of silica in the material to be analyzed may reduce the recovery of fluorine during distillation. The amount of fluorine recovered from grass hay decreased with increasing amounts of silica when the sodium hydroxide fusion was not used (1). Because the silica content of plants might vary considerably, the caustic fusion of the ash was always employed.

Direct fusion of plant material with sodium peroxide in a Parr bomb may also be used. In several species of plants over a wide range of fluorine levels, the results from procedures using sodium peroxide fusion and calcium oxide ashing have shown good agreement (7). The sodium peroxide fusion is quicker than the calcium oxide ashing, only 30 minutes compared to 6 hours, and smaller samples are used (0.5 gram).

Distillation. The cooled melt is dissolved in a minimum amount of water and transferred quantitatively to the distillation flask in the steam-distillation apparatus shown in Figure 3. The total volume of water used should not exceed 75 ml. About 0.5 gram of glass wool is then added. Fifty milliliters of 70 per cent perchloric acid is added, providing an excess over the portion neutralized by the melt. One to 2 ml. of 25 per cent silver perchlorate solution is added to prevent the carryover of halides other than fluorides. The final volume of the solution in the distillation flask should not exceed 150 ml.

The hot plate under the steam generator and the mantle around the distillation flask are turned on and the distillation starts at 110° C. When the temperature reaches 130° C., steam is admitted from the generator by closing the Hoffman clamp (Fig. 3). The temperature is maintained at 135° ± 2° C. by adjustment of the Powerstat connected to the mantle. A volume of 450 to 500 ml. of distillate is collected in polyethylene bottles, and the distillation is usually completed in 75 minutes. After the distillate is adjusted to 500 ml., it is ready for titration. The distillate may also be stored for several days, preferably in a cold room (1).

Titration. A photoelectric filter photometer previously described (1, 6) is used for end-point determination in the direct titration of fluorides with thorium nitrate. The blank contains 250 ml. of fluorine-free distilled water adjusted to pH 3.0 ± 0.05 and 2 ml. of sodium alizarinsulfonate indicator. An equal volume of distillate is placed in the sample cell and adjusted to pH 3.0 ± 0.05, and 2 ml. of indicator is added. The cells are placed in the photometer, the light is turned on, and the system balanced by iris diaphragms until the galvanometer spot rests at mid-scale. The Ayrton shunt is switched to the desired sensitivity. For an expected fluorine content up to 100 μg., x1 (maximum sensitivity) is used; for amounts up to 1,000 μg. position x10 is used. The sample is titrated dropwise from a microburette

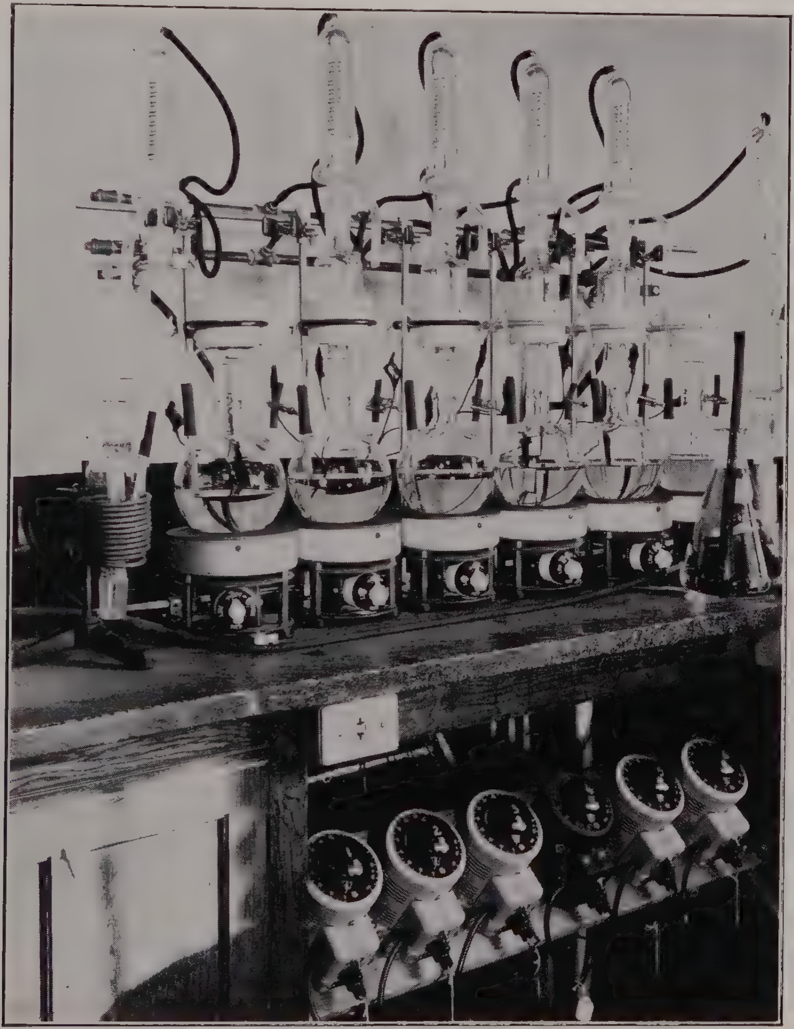

FIGURE 3. Assembly set of six steam-distillation flasks, shown in various stages of operation.

containing standard thorium nitrate solution. The tip of the microburette enters the top of the sample chamber, and a magnetic stirrer is used to ensure rapid and complete mixing. The titration is complete when the galvanometer spot is deflected an arbitrary number of units from its initial position (a scale reading of 25 was usually chosen). The volume of thorium nitrate solution required to produce this deflection in a fluorine-free sample is deducted from the amount used in the titration. The fluorine content of the sample is obtained from the volume of thorium nitrate used by reference to a calibration curve prepared with known amounts of fluoride. The thorium nitrate solutions used are 0.01N and 0.00145N for fluoride contents up to 1,000 and 100 µg., respectively. The light source of the photometer can be connected directly to the 115-volt line without using a voltage stabilizer if a 0.001N thorium nitrate solution is used and the voltage varies within 5 per cent. If the voltage variation is greater than 5 per cent or a 0.01N thorium nitrate solution is used, a voltage stabilizer is recommended (8).

Water analysis. The volume of water is reduced to about 5 ml. in the presence of calcium oxide under the infrared lamp and transferred quantitatively to the distillation flask. Samples of 50 ml. or less may be steam distilled directly and titrated by the above procedures.

Air analysis. A volume of 40 to 70 ml. of fluorine-free water is added to the absorber. The absorber is placed in the area to be sampled and connected to a vacuum line through an air meter. The minimum volume of air to be drawn depends upon the concentration of fluorine expected and the individual operator. Usually 40 to 50 cu. ft. is needed at concentrations of 10 p.p.b. and 5,000 cu. ft. or more at 0.1 p.p.b. The air is passed through the absorber at a rate of 20 to 40 cu. ft. per hour and should not exceed 50 cu. ft. per hour. The sampling is continued over several days when low fluoride concentrations are expected, and the water must be replenished and a constant level maintained either manually or automatically by the device shown in Figure 4. By placing the absorbers in series, it was found that 90 per cent of the HF was absorbed in the first vessel when air containing 12.7 to 30 p.p.b. HF was drawn at a maximum rate. Single absorbers placed in a fumigation chamber showed a coefficient of variation of 4.3 to 5.6 per cent.

When the sampling is completed, the absorption solution is transferred quantitatively from the absorber to the titration cell of the photoelectric titrator. The volume is brought to 250 ml. and the pH adjusted to 3.0 ±0.05 and the titration carried out by the above procedure. When sulfur dioxide or other substances which may interfere with titration are present in the sample, the absorbed fluorine must be steam distilled. For this purpose the absorber shown in Figure 5 is used. This absorber includes the essential features of the Greenburg-Smith impinger and allows the absorbed fluorides to be steam distilled directly without handling or transfer of samples to a separate distillation apparatus. Forty milliliters of 70 per cent perchloric acid is added to the absorber which is then connected to a condenser and steam generator. The distillation follows the above procedure except that only 250 ml. of the distillate is collected. Steam distillation must also be used when particulate matter is involved (1).

The absorption-distillation unit used in recent years, the new absorption-distillation impinger unit, and the Greenburg-Smith impinger were

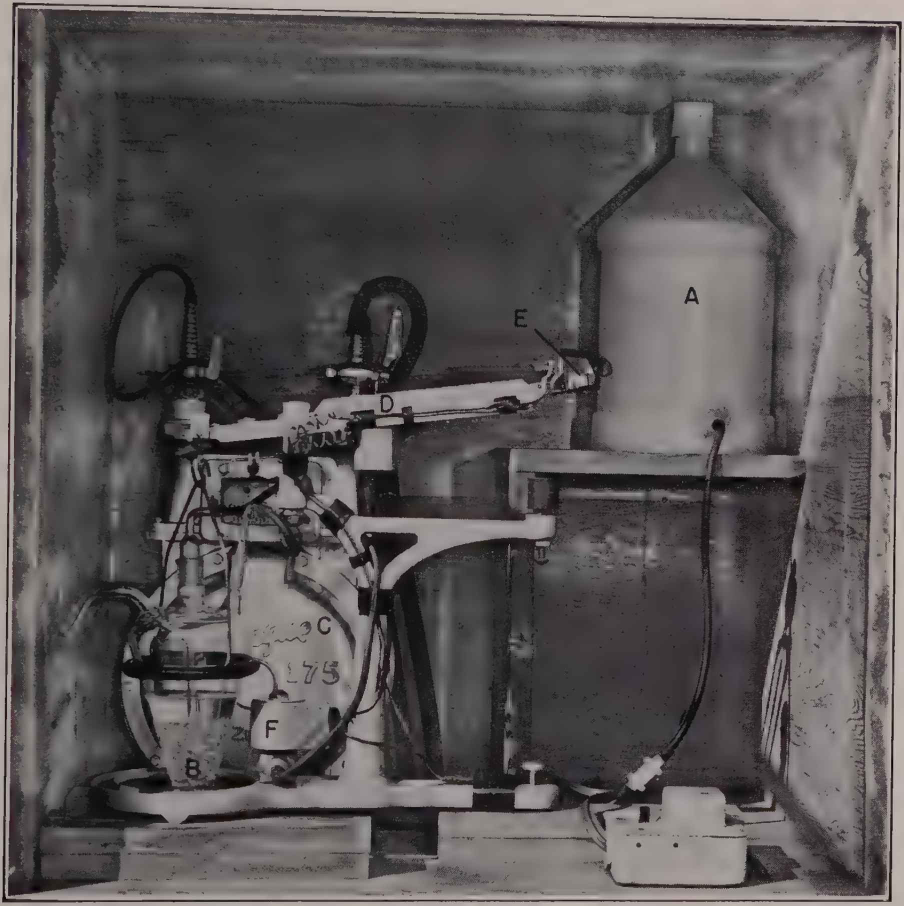

FIGURE 4. Apparatus used for continuous absorption. A. Water reservoir; B. absorber; C. air meter; D. balance; E. mercury switch; F. solenoid valve. After 30 ml. of water has been evaporated, the balance arm swings down, the mercury switch opens the solenoid valve, and water flows into the absorber from the reservoir.

compared with respect to their reliability in detecting various concentrations of different types of atmospheric fluorides. The absorbers were placed together in a chamber in which different types of fluorine compounds were added to the air stream. On different occasions tests were made with HF at about 8 p.p.b., sodium fluoride aerosol at 8 to 10 p.p.b., particulate cryolite at 9 to 180 p.p.b., and particulate material from electrostatic precipitators at 5 to 20 p.p.b. There were no significant differences among the results obtained with the three types of absorbers except where particulate cryolite was used at 180 p.p.b. There the Greenburg-Smith impinger showed a significantly higher concentration than the other absorbers (Table II). The

Greenburg-Smith impinger gives efficient collection of aerosols and particulate matter down to a particle size of 0.5 μ diameter (9). Since the Greenburg-Smith impinger was used as a reference, the efficiency of the absorption units tested must be limited to the same range of particle sizes.

Reliability of sampling methods. In the analysis of vegetation the methods used in sampling constitute an important part of the entire procedure. In alfalfa exposed to 1 to 6 p.p.b. of HF for 8 days, the concentration (p.p.m.) of fluorine in new growth was 4 to 5 per cent of that in the older growth. The level of fluorine in fruit of fumigated tomato (*Lycopersicon esculentum* Mill.) plants was 1 per cent of that in the leaves (11).

Fluorine is accumulated most readily in the tips and margins of leaves. In Golden Cross Bantam sweet corn (*Zea mays* L.), exposed for one week to an atmosphere containing 12 p.p.b. HF, the upper halves of the leaves had 167 p.p.m. and the lower halves 44 p.p.m. of fluorine. The tips of *Gladiolus* leaves with necrotic streaks consistently showed higher fluorine concentrations than the basal parts of the leaves. Seven varieties had fluorine levels of 49 to 218 p.p.m. in the terminal 4 inches, but the subjacent 4 inches ranged from 21 to 37 p.p.m. after prolonged fumigation with HF. The margins of leaves of peach (*Prunus* sp.), walnut (*Juglans* sp.), ash (*Fraxinus* sp.), and grape (*Vitis* sp.) had 20 to 1,400 per cent higher fluorine levels

TABLE II

Comparison of the Reliability of the Conical Absorption-distillation Unit (A), a Newly Developed Conical Absorption-distillation Impinger (B), and the Greenburg-Smith Impinger (C) for Determining the Concentration of Gaseous and Particulate Fluorine Compounds in the Atmosphere of Fumigation Chambers. Each Compound Was Used in Three Different Tests in Which the Three Absorbing Units Were Placed Side by Side. Fluorine Concentration Expressed as P.P.B. v/v of HF. All Air Samples Were Steam Distilled

Compound used	Absorbing unit		
	A	B	C
HF gas	8.1	8.4	8.8
	6.9	7.8	7.7
	8.4	7.7	7.9
Sodium fluoride aerosol	9.7	10.3	10.1
	10.3	10.0	8.5
	8.5	7.7	9.7
Particulate cryolite	162	158	183*
	8.8	8.5	8.7
	9.9	8.9	11.5
Particulate material from electrostatic precipitators	19.8	21.1	24.3
	13.9	15.1	15.8
	5.2	5.6	5.3

* Significantly higher than A or B (5 per cent level).

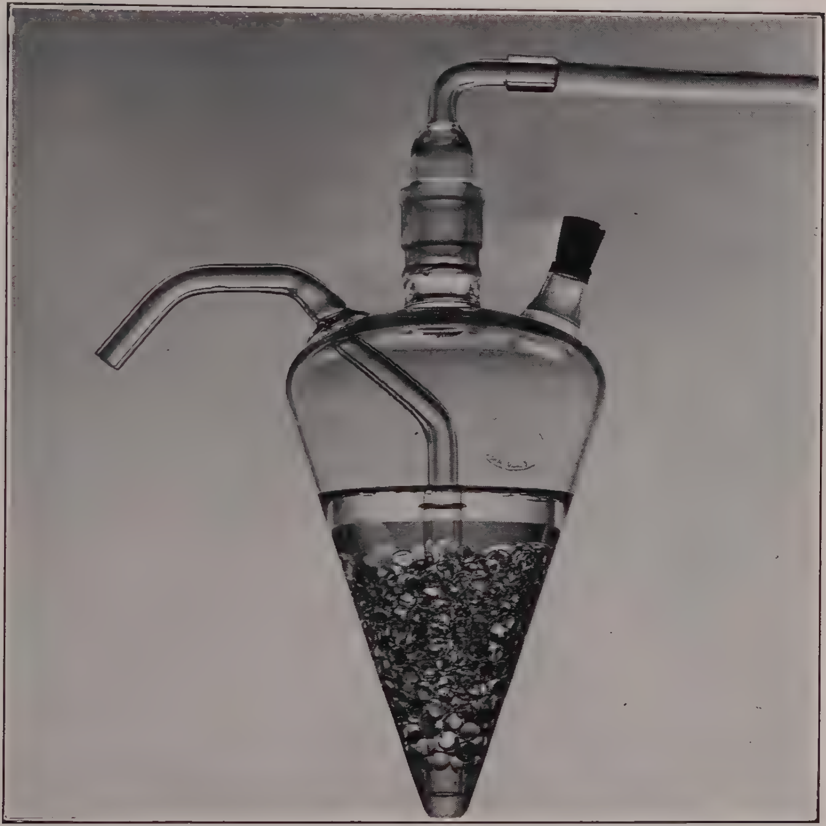

FIGURE 5. Five hundred milliliter absorber. When the outlet in the center is connected to the condenser, the tube on the left connected to the steam generator, and a thermometer put in place of the rubber stopper, the absorber is converted to a steam-distillation flask.

than the central areas of the leaf after exposure to 6.8 p.p.b. HF for 7 days (11).

Both the concentration and total amount of fluorine in leaf tissues decrease after fumigation. In leaves of tomatoes grown in pots, the fluorine concentration decreased from 650 to 250 p.p.m. and the fluorine content decreased from 350 to 50 μg. fluoride per plant over a 9-day period after an exposure of 9 days to an atmosphere with 10 p.p.b. of HF. In gladiolus var. Snow Princess containing 81 to 129 p.p.m. fluorine, the fluorine concentration dropped 50 per cent over a 2-week period.

Thus, caution must be used in interpreting analytical data on the fluorine status of a plant until the method of sampling is clearly defined. Data may be misleading if based solely on one part of a leaf, one or two leaves, or an entire organ.

Any one analysis of tissues during a growing season may not provide a complete picture of the fluorine status of a plant or the potential capacity for damage to the crop but indicates that the plant has been exposed to fluorides of atmospheric or soil origin at some time in its history.

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BIOCHEMICAL STUDIES OF DORMANCY AND AFTER-RIPENING OF SEEDS. III. NITROGEN METABOLISM[1]

LELA V. BARTON AND JANE L. BRAY

SUMMARY

Nitrogen metabolism of tree peony endosperm and embryo tissues during the after-ripening of the dormant epicotyl at 5° C. was compared with that of controls kept in the greenhouse (ambient temperature of 21° to 30° C.) where the epicotyl remained dormant.

Of the free amino acids or amides, alanine and glutamine were present in larger quantities in tissues of seedlings held at 5° C. than in those in the greenhouse. Asparagine was more abundant in tissues held at the higher greenhouse temperature.

Total protein content of endosperm tissues decreased after the seedlings were planted in soil with a more rapid decline after 8 and 12 weeks at 5° C. than at greenhouse temperature. In embryo tissue, on the other hand, there was a steady increase in protein content upon germination and growth for several weeks in soil, with greater protein synthesis after 8 and 12 weeks at 5° C. than at greenhouse temperature.

The ten most abundant amino acids in protein hydrolyzates were alanine, arginine, aspartic acid, glutamic acid, glycine, leucine, serine, threonine, tyrosine, and valine. Aspartic and glutamic acid components increased markedly in after-ripened embryos. No significant alteration was noted in corresponding endosperm tissue. On the other hand, endosperm tissue associated with dormant embryos contained increased percentages of aspartic and glutamic acids.

INTRODUCTION

A study is being made of the changes in chemical composition in relation to the after-ripening of the dormant epicotyl of the tree peony (*Paeonia suffruticosa* Haw.). Biochemical analyses permit comparisons of tissues held in the greenhouse, where they remain dormant, with those held at 5° C. where after-ripening takes place. Two publications have dealt with changes in free amino acids, oligobasic organic acids, and carbohydrates (1, 2).

Further studies of nitrogen metabolism during after-ripening are the subject of the present report.

MATERIAL AND METHODS

The plant material used consisted of residues of ground samples of tree peony seedlings in various stages of after-ripening as prepared for determination of oligobasic organic acids and carbohydrates (1).

Free amino acids were determined by the method already described (2).

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Semi-micro Kjeldahl analyses were used for protein nitrogen determination and the amount of protein was calculated by assuming that the proteins contain 16 per cent nitrogen. Proteins were hydrolyzed with $6N$ HCl, dried and stored at 1° C. until they were taken up in 10 per cent isopropanol for spotting on chromatograms. Chromatographic analyses of all amino acids were made according to the method of Porter *et al.* (6).

RESULTS AND DISCUSSION

Free Amino Acids

Although determinations of free amino acids were made earlier on this same type of material (2), they were repeated for representative lots using some of the original samples as prepared for the present series of tests. This was felt to be necessary in order to corroborate previous findings and to give a more complete analysis of nitrogen metabolism.

In general the same results were obtained as previously. In the endosperm tissue, seedlings maintained at 5° C. had more alanine than in those held in the greenhouse, especially after eight weeks when amounts at 5° C. were four to five times those in the greenhouse. Glutamine in endosperm from 5° C. was 10 times that in the greenhouse after one week and thereafter continued to be significantly higher. On the other hand, asparagine was in greater supply in endosperm tissues in the greenhouse. The embryo tissues followed the same pattern as those of the endosperm as regards alanine, glutamine, and asparagine.

Analyses of free amino acids of root and shoot portions of the germinated embryo were also made after 0, 2, and 12 weeks at 5° C. or in the greenhouse. The roots contained more glutamine than the shoots at the time of planting and after 2 weeks at 5° C. but not after 12 weeks when the shoots had a larger quantity. This was to be expected since the shoot is the organ in which dormancy is broken and since glutamine accumulation may be related to after-ripening. More glutamine was present in both roots and shoots held at 5° C. than in those in the greenhouse. Similarly, a temperature of 5° C. resulted in higher alanine content than did greenhouse temperature in both roots and shoots. The shoots contained more alanine than roots at the time of planting and after 2 weeks, but this difference was not obvious after 12 weeks. Thus, limited tests (after 0, 2, and 12 weeks only) on the component parts of the embryo confirmed the findings for the entire embryo for free amino acid contents.

Total Proteins

As has been found in the case of other constituents of tree peony seeds and seedlings (1, 2), protein contents expressed on the basis of dry weights of the samples are misleading as far as their possible role in growth is concerned. It will be noted (Table I) that the milligrams of protein per 100 mg.

of dry weight varied little with endosperm or embryo portions or with treatment. Dry weights of endosperm tissues from twenty dry seeds averaged 1.52 g. (1). This declined gradually to 0.82 g. as the stored food was used by the developing embryo. Embryo weights increased gradually from 0.0058 g. for those from twenty dry seeds to 0.494 g. for twenty seedlings after twelve weeks in soil, paralleling the decrease in endosperm weights.

When one considers the amount of protein in 20 endosperm or embryo portions of seeds or seedlings, certain trends become evident. Protein content of endosperm tissues from twenty seeds or seedlings decreased for several weeks after planting in soil with a greater decline after 8 and 12 weeks at 5° C. than at greenhouse temperature (Table I). In embryo tissue there was a steady increase in protein content upon germination and with progressive periods of 1 to 12 weeks after germination and planting in soil (Table I). In contrast to endosperm tissue, embryos contained more protein after 8 and 12 weeks at 5° C. than at greenhouse temperature.

Quantitative measurements of protein were made of samples of root and shoot portions of the embryos of germinated seedlings and of seedlings held for 12 weeks in soil in the greenhouse or in a room at 5° C. Roots were severed just below the enlarged hypocotyl, which, together with the epicotyl and the cotyledons, were classed as "shoots." Twenty germinated

TABLE I

AMOUNT OF PROTEIN IN ENDOSPERM AND EMBRYO TISSUES OF TREE PEONY SEEDS AND SEEDLINGS

Treatment		Protein content of tissues			
		Endosperm		Embryo	
Temp.	Time (wks.)	Mg./100 mg. dry wt.	Mg./20 seeds or seedlings	Mg./100 mg. dry wt.	Mg./20 seeds or seedlings
Dry seeds*		25.1	381.5	31.6	1.9
Imbibed seeds*		28.8	381.7	33.1	1.3
Germinated seedlings*		19.8	352.4	26.8	8.3
Greenhouse (21° to 30° C.)	1	25.4	449.6	26.5	15.4
	2	26.4	438.2	18.2	16.2
	4	29.1	468.5	21.8	27.9
	8	25.8	394.7	22.4	55.8
	12	29.8	336.7	28.3	93.7
5° C.	1	26.3	470.8	31.5	14.5
	2	25.9	455.8	25.7	14.9
	4	25.8	433.4	30.2	26.3
	8	28.8	357.1	23.0	80.7
	12	31.1	255.0	27.4	135.4

* Analyses were made of dry seeds, seeds which had imbibed water but had not germinated, and germinated seedlings as used for planting in soil.

seedlings at the time of planting in soil contained 2.3 mg. and 6.0 mg. of protein in root and shoot tissue respectively. Protein content of the roots increased after 12 weeks in soil, but showed no differences due to temperature treatment (49.1 or 47.6 mg. 20 roots, held in the greenhouse or at 5° C.). Protein synthesis took place in shoots during 12 weeks in the greenhouse (24.7 mg./20 shoots) with a still greater increase after the same time at 5° C. (64.2 mg./20 shoots).

It has been determined (2) that the root system extends markedly in soil in the greenhouse, while it remains short but thickens at 5° C. The dry weight of roots from 20 embryos held in soil in the greenhouse or at 5° C. for twelve weeks was 0.157 g. and 0.186 g. respectively. Since the tissue weights were approximately the same at the two temperatures, it is not surprising that the amount of protein was similar.

The dry weight of shoot portions of 20 embryos, on the other hand, was only 0.123 g. after 12 weeks in soil in the greenhouse as compared to 0.303 g. after the same period at 5° C. (2). The shoot contains the dormant organ, the epicotyl, and metabolic activity of this organ, including protein synthesis, undoubtedly accompanies after-ripening at 5° C. resulting in a significant increase in weight.

Amino Acid Composition of Protein

Starch was not removed from protein samples before hydrolysis since preliminary experiments indicated no additional humin formation in the presence of starch. It should be noted that hydrolysis alone destroyed all tryptophan and may have destroyed some arginine, histidine, lysine, methionine, and tyrosine.

Ten of the amino acids detected in protein hydrolyzates (alanine, arginine, aspartic acid, glutamic acid, glycine, leucine, serine, threonine, tyrosine, and valine) accounted for a major portion of the protein amino acids. Four other amino acids (cysteic acid, proline, lysine, and phenylalanine) were detected in very small quantities or in trace amounts. Other ninhydrin-positive constituents were not identified.

The amounts of the ten most abundant amino acids produced by protein hydrolysis, expressed as milligrams per 20 seeds or seedlings, are shown in Table II. With changes in actual quantities of protein during metabolism of the tree peony seedling under different temperatures, there occurred differences in the relative amount of component amino acids, as expressed by the percentages of total amino acids determined. A comparison of these differences after eight weeks, the period required for after-ripening at 5° C., may indicate some metabolic effects of temperature.

In embryo tissue, for example, eight weeks at 5° C. resulted in decreased percentages (over germinated seedlings) of arginine (16 to 7 per cent), leucine (11 to 4 per cent), alanine (9 to 7 per cent), threonine (10 to 2 per

TABLE II

CHANGES IN AMOUNTS OF THE TEN MOST PREVALENT AMINO ACIDS IN PROTEINS OF ENDOSPERM, EMBRYO, ROOTS, AND SHOOTS OF DORMANT (GREENHOUSE) AND AFTER-RIPENING (5° C.) TREE PEONY SEEDS AND SEEDLINGS OVER A 12-WEEK PERIOD

Treatment		Amount of amino acid (mg./20 seeds or seedlings)									
Temp.	Time (wks.)	Alanine	Arginine	Aspartic acid	Glutamic acid	Glycine	Leucine	Serine	Threonine	Tyrosine	Valine
		Endosperm									
Dry seeds*		6.7	10.0	16.7	20.0	10.0	10.0	10.0	13.3	10.0	5.0
Imbibed seeds*		8.6	6.9	12.0	18.9	10.3	10.3	10.3	6.9	Trace	5.2
Germinated seedlings*		9.2	9.2	11.0	18.3	11.0	9.2	9.2	7.3	Trace	3.7
Greenhouse (21°–30° C.)	1	15.1	21.9	8.4	37.0	18.5	20.2	13.5	8.4	1.7	10.1
	2	17.9	23.3	26.9	44.8	16.1	26.9	16.1	12.5	5.4	10.8
	4	17.1	10.3	6.9	41.1	18.9	27.4	15.4	12.0	5.1	12.0
	8	13.5	13.5	44.0	67.6	23.7	10.1	23.7	8.5	1.7	8.5
	12	14.4	18.3	18.3	38.0	15.7	22.3	14.4	10.5	7.9	9.2
5° C.	1	13.9	17.4	8.7	31.4	15.7	15.7	10.5	3.5	3.5	7.0
	2	14.0	15.7	14.0	35.0	15.7	17.5	10.5	10.5	1.7	8.7
	4	14.8	8.2	11.5	32.8	16.4	18.0	11.5	8.2	4.9	6.6
	8	17.3	20.1	18.7	40.3	15.8	24.4	17.3	12.9	12.9	11.5
	12	9.8	6.8	6.8	20.4	9.1	17.4	9.1	7.5	3.8	7.5
		Embryo									
Dry seeds*		0.06	0.08	0.04	0.10	0.07	0.09	0.06	0.05	0.02	0.05
Imbibed seeds*		0.05	0.03	0.04	0.05	0.06	0.08	0.05	0.04	0.02	0.03
Germinated seedlings*		0.59	1.1	0.63	0.82	0.56	0.78	0.59	0.71	0.74	0.45
Greenhouse (21°–30° C.)	1	0.74	1.8	0.61	0.86	0.55	0.80	0.68	0.55	0.18	0.43
	2	0.64	0.54	0.54	0.54	0.54	0.54	0.32	0.22	0	0.32
	4	1.3	2.4	1.5	1.5	1.3	2.4	1.3	1.1	0.38	0.76
	8	2.1	5.2	2.1	2.9	2.1	2.6	2.1	1.8	1.3	1.3
	12	2.6	7.3	3.2	4.4	3.5	4.1	3.8	2.9	2.6	2.0
5° C.	1	0.59	1.1	0.49	0.78	0.49	0.78	0.54	0.49	0.29	0.34
	2	1.1	2.0	0.83	1.3	0.45	1.6	0.89	0.83	0.38	0.64
	4	1.2	2.3	1.4	1.5	1.0	1.8	1.1	0.93	0.47	0.93
	8	2.2	2.2	11.7	6.9	2.9	1.5	2.9	0.73	0.37	1.1
	12	4.1	10.1	4.1	5.5	3.7	6.0	3.7	3.2	?	2.8
		Roots									
Germinated seedlings*		0.21	0.56	0.33	0.27	0.12	0.25	0.25	0.19	0.06	0.14
Greenhouse	12	1.7	3.9	2.7	3.0	1.6	2.4	2.4	1.7	1.4	1.4
5° C.	12	1.7	4.7	1.9	2.4	1.9	3.2	1.9	1.7	0.93	1.5
		Shoots									
Germinated seedlings*		0.43	0.88	0.55	0.54	0.23	0.80	0.45	0.43	0.23	0.32
Greenhouse	12	0.86	2.1	0.86	1.2	0.74	0.99	0.99	0.86	0.25	0.62
5° C.	12	2.7	5.8	3.0	3.0	2.7	3.6	2.4	2.4	0.61	2.1

* See footnote in Table I.

cent), valine (6 to 3 per cent), and tyrosine (11 to 1 per cent); and greatly increased proportions of glutamic (12 to 21 per cent) and aspartic (9 to 36 per cent) acids. Eight weeks at greenhouse temperature, on the other hand, resulted in a threefold increase in quantity of the ten most abundant amino acids but still showed the same proportional relationship among the amino acids with the possible exception of tyrosine, the percentage of which was reduced (from 11 to 6).

In contrast to the composition of proteins in the embryo, the endosperm tissue after eight weeks at 5° C. contained approximately the same percentages of eight of the ten amino acids, with increases noted only for valine (4 to 6 per cent) and tyrosine (trace to 7 per cent). After eight weeks in the greenhouse, however, the percentages of glutamic (21 to 31) and aspartic (12 to 20) acids increased significantly in endosperm tissue.

Root and shoot portions of the embryo were analyzed in the germinated seedlings and in seedlings held for twelve weeks in soil at greenhouse temperature and at 5° C. Percentages of component amino acids in these portions agreed closely with those in the entire embryo for this period, which extended four weeks beyond the period required for after-ripening at 5° C.

GENERAL DISCUSSION

It should be kept in mind that dormancy of the tree peony epicotyl is broken at 5° C., so that, upon subsequent transfer to the greenhouse, the green shoot appears above the soil and continues to grow. However, only occasional green shoots appear above ground in the greenhouse without low-temperature pretreatment, and the underground portions of the seedlings finally die. Thus, normal metabolism and growth follows after-ripening at 5° C., while tissues held dormant at greenhouse temperatures gradually deteriorate.

The possible significance of the larger quantities of free alanine and glutamine in tissues of tree peony seedlings held at 5° C. and the corresponding larger quantities of asparagine in tissues of seedlings held at greenhouse temperatures has been discussed in a previous paper (2). Germination studies by other authors have offered possible explanations for these differences. For example, external application of glutamine at 10 mg./l. stimulated growth of plant embryos of 12 species *in vitro* (9). Asparagine also stimulated germination at 10 mg./l. but became inhibitory at higher concentrations, especially in the absence of glutamine, and caused very little growth in low concentrations. Rijven (9) points out that if the asparagine enzyme system contributes little to synthesis, then a large surplus of external asparagine may block the main glutamine pathway thus effecting an over-all growth depression.

Aspartic and glutamic acid components of protein in tree peony tissues may also be related to dormancy. The proportion of both of these acids increased with after-ripening of the embryos, while no significant alteration was noted in corresponding endosperm tissue. On the other hand, endosperm tissue associated with dormant embryos contained increased percentages of aspartic and glutamic acids. These findings indicate the utilization of these acids by the after-ripening embryo and the failure of the dormant embryo to use the reserves available in the endosperm.

Sivaramakrishnan and Sarma (10) investigated the metabolism of C^{14}-labeled glutamic acid, and found strong evidence for its synthesis during germination of seeds of *Phaseolus radiatus*. They considered aspartic acid and asparagine to be products of glutamic acid degradation. The use of C^{14}-labeled glucose indicated conversion from carbohydrates to glutamic acid. They concluded that rapid degradation and extensive synthesis of glutamic acid during germination clearly indicate active metabolism in germinating seeds.

There is no doubt that glutamic acid, aspartic acid, and alanine are of prime importance in the nitrogen metabolism of plants (3, 4, 11). Glutamic acid furnishes the nitrogen of alanine and aspartic acids and forms the carbon skeleton of arginine, cysteine, cystine, glycine, hydroxyproline, proline, and serine. Of the natural L-amino acids only three, alanine, aspartic acid, and glutamic acid, are formed by the amination of α-ketoacids, i.e. pyruvic, oxaloacetic, and α-ketoglutaric. Rabson (7) and Rabson *et al.* (8) consider that the point of contact between ketoacid and amino acid metabolism is to be regarded as the focus for the effects of environment upon metabolism. He also stated (7) that of the soluble amino acids in mint plants, glutamic and aspartic acids, and their amides, glutamine and asparagine, show the greatest change in response to environmental changes. Low night temperatures favor accumulation of glutamic acid and glutamine while high night temperatures increase the level of γ-aminobutyric acid and asparagine.

The greater proportion of arginine in the endosperm and the smaller quantity in the embryo of tree peony after eight weeks at 5° C. indicate that this amino acid may be less important in further embryo growth. Zacharius *et al.* (12) have shown the accumulation of arginine when protein synthesis is arrested in the tulip bulb.

At temperatures above those at which after-ripening proceeds (above 10° C.) respiration of dormant seeds rises markedly according to Nikolaeva and Katkevich (5), thus preventing the accumulation of hydrolysis products necessary for germination. No respiration measurements of tree peony seedlings have been made.

It is evident that exposure to cold increases the growth potential of the tree peony embryo. This may be accomplished by conversion of the stored food of the endosperm to intermediates required for growth. It is also possible that some product of the embryo at low temperature may diffuse into the endosperm and break down storage proteins into the amino acids which promote growth. Similarly, metabolic inhibitors may be produced in the endosperm or embryo or both at the higher temperature of the greenhouse. Further studies are needed to elucidate the mechanism by which low temperature promotes growth.

Still to be reported in the current series of investigations are analyses of tissues of tree peony seedlings for the possible relation of growth regulators to dormancy and after-ripening.

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THE BEAN RUST METHOD OF EVALUATING FUNGICIDES IN GREENHOUSE TESTS

S. E. A. McCallan and Rina C. Zingerman

SUMMARY

Factors influencing infectivity of bean rust, *Uromyces phaseoli* (Pers.) Wint., and the precision of the method for evaluating protectant fungicides were studied on the variety Pinto (*Phaseolus vulgaris* L.). No consistent difference was found in the homogeneity of infection of replicate potted plants inoculated by spray atomization or by dust application of the uredospores. However, while the number of infections was more or less proportional to the number of spores in spray atomization, in dust application the infections tended to be proportionately less as the number of spores increased. Talc was superior to six other carriers in improving the handling and wettability of the spores for inoculation by atomization. Freshly harvested spores stored in vials at temperatures from 0.5° to 22° C. will maintain some infectivity up to 8 weeks. The lower temperature storage prolonged infectivity relatively little.

A series of dosage tests with 10 standard sulfur and organic fungicides was run at 9 different times. The regression of the logarithm of the weight (reciprocal of variance of replicate plants) on logarithm of mean percentage of disease did not differ significantly with those for early and late blights of tomato or snapdragon rust previously reported. Thus the most precise level of comparison within a test is also approximately in the range 80 to 95 per cent disease control. Five fungicides (wettable sulfur, thiram, nabam, glyodin, and captan) gave orthodox straight line dosage-response curves when plotted as probits against logarithm of concentration, but the other 5 (lime sulfur, ferbam, maneb, zineb, and dichlone) showed highly significant curvature. The fungicide × replicate test interaction at the ED95 level is the appropriate error term for comparing fungicides. The magnitude of this indicates that the bean rust method is less precise than that of late blight of tomato, but more precise than that of early blight of tomato or snapdragon rust.

INTRODUCTION

Bean rust has been commonly used as a means of evaluating the effectiveness of fungicides in greenhouse tests because it is a representative of the rust diseases which is simple to handle. However, there is little or no information in the literature on the various factors involved and on the precision of the method. Accordingly, some of the factors have been studied and in particular the precision has been determined with a series of standard fungicides.

MATERIALS AND METHODS

The bean rust, *Uromyces phaseoli* (Pers.) Wint.,[1] was cultured on bean plants (*Phaseolus vulgaris* L.) var. Pinto grown in 5-inch pots. The plants

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[1] Obtained originally from Dr. C. E. Yarwood, University of California, Berkeley, California.

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were used about 10 days after planting the seed when they had attained a height of some 6 inches. The day before use they were thinned to 3 plants per pot by breaking or cutting off the tops of the excess plants.

The spores for inoculation were obtained from prior experiments and were usually stored in vials held in a refrigerator. The plants were inoculated either by atomizing a water suspension of the spores on the plants which were held on a turntable in the usual standard procedure (4, 5, 8) or the dry spores diluted with a carrier dust were applied in a vacuum duster. A special large aluminum alloy vacuum duster was used for this purpose. The same loading device was employed as for the original bell jar duster(6). The alloy duster was cast in two parts, the cylinder and dome, which were bolted together by means of external flanges. The internal diameter is 20 inches and height 42 inches. The duster was usually evacuated to 10 lbs. of pressure. Up to eight 4-inch pots of bean plants may be accommodated. The plants were exposed for 2 minutes after releasing the spores. After inoculation the plants were held in walk-in infection chambers (7) at 60° F. for 21 to 24 hours. The rust pustules on the 6 primary leaves in each pot were counted about 10 days later.

The standard fungicides used in the tests were lime sulfur; wettable sulfur (Sulforon,[2] 97 per cent sulfur); thiram (Thylate,[2] or Arasan,[2] respectively 65 per cent and 50 per cent tetramethylthiuram disulfide); ferbam (Fermate,[2] 76 per cent ferric dimethyldithiocarbamate); nabam (Dithane D-14,[3] 19 per cent disodium ethylenebis[dithiocarbamate]); zineb (Parzate,[2] 65 per cent zinc ethylenebis[dithiocarbamate]); maneb (Manzate,[2] 70 per cent manganese ethylenebis[dithiocarbamate]); dichlone (Phygon,[4] 50 per cent 2,3-dichloro-1,4-naphthoquinone); glyodin (Crag Glyodin,[5] 34 per cent 2-heptadecyl-2-imidazoline acetate); and captan (Captan 50W,[6] 50 per cent *N*-[trichloromethylthio]-4-cyclohexene-1,2-dicarboximide). These were applied in a dosage series by means of the standard spray paint gun procedure (5).

RESULTS

Method of inoculation. As in all bioassay methods it is desirable to reduce the variation of replicate plants and replicate tests as much as possible in order to be able to show smaller significant differences. Comparisons were made of inoculations with spores in suspension in water and of dry spores in the vacuum duster. Since rust spores do not wet readily, talc was added in both methods. These comparisons were made in factorial tests in association with other variables and the spores were applied at 3 concentration levels. Because of the wide range in numbers of lesions all analyses of results were made on the logarithms of the number of lesions per pot of 3 plants. In one test the spray inoculation appeared to give more homogenous infection, in another the dust inoculation was superior, and in the third there was no significant difference. These results with replicate plants in the three different tests with 102 degrees of freedom for each gave a variance of 0.0114 and 0.0110 for the spray and dust inoculated plants, respectively. Obviously there is no consistent difference in the variability of replicate plants inoculated by either method.

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[2] E. I. du Pont de Nemours and Co., Inc., Wilmington 98, Delaware.

[3] Rohm & Haas Co., Philadelphia 5, Pennsylvania.

[4] United States Rubber Co., Naugatuck Chemical Div., Naugatuck, Connecticut.

[5] Union Carbide Chemicals Co., New York 17, New York.

[6] Stauffer Chemical Co., New York 17, New York.

The relation of spore concentration of inoculum to number of rust pustules is shown in Figure 1. In this more or less typical test spray atomization is based on 20 ml. of spore suspension per turntable of 4 pots of plants, with talc added at a rate of 80 mg. per 2 mg. of spores. Inoculations in the vacuum duster were with mixtures of spores and talc in the respective ratio of 10 mg. to 200 mg., applied to 8 pots of plants. It will be seen that the number of rust pustules is approximately proportional when the inoculation is made with spores suspended in water, as has been found for other greenhouse diseases (8). However, with the inoculation using dry spores in a dust chamber, the curve on this logarithmic scale has a flatter slope, i.e. less than 1.0, hence there tend to be proportionately less infections as the number of spores is increased.

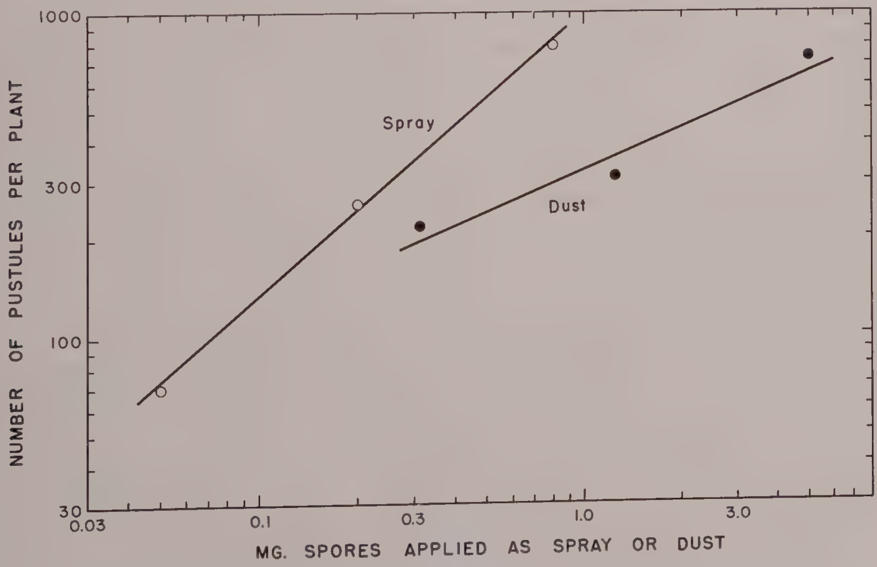

Figure 1. Relation between number of bean rust pustules per plant and number of spores applied by spray atomization or by dust application.

Carriers. The commonly used carrier, talc, was compared with 6 other carriers which are described in detail in an earlier paper (6) and listed in Table I. The carriers were used at rates of 20, 80, and 320 mg. per 3 mg. of spores suspended in 200 ml. of water and the plants inoculated by atomizing. There were 4 replicate pots per concentration. Two of the carriers, wood flour and Cherokee clay, tended to clog the atomizer at the higher concentrations. An analysis of variance (12) indicated that there was a highly significant difference between the remaining carriers in the amount of infection and the greatest amount was obtained with talc as may be seen in Table I. However, the Duncan test (1, 2) indicates that the infection using talc is significantly greater than with all other carriers, and that with bentonite and Celite infections are greater than with Attaclay. It is also evident that infectivity does not increase in proportion to the amount of carrier. There is, however, a trend toward a linear increase in infectivity with the logarithm of the concentration of carrier.

Effect of storage temperature and time on infectivity of uredospores. It is recognized that the uredospores of bean rust rapidly lose their infectivity when harvested and held at room temperature and that infectivity may be prolonged by holding in a refrigerator. For long periods of storage the lyophilizing procedure would be essential (11). However, a simpler process would be desirable to insure a supply of spores for week-to-week needs. Accordingly, spores were stored immediately after harvesting in stoppered glass vials at temperatures of 21°, 18°, 15°, 10°, 5°, and 0.5° C. and plants inoculated after varying periods of time. A series of different tests was made which covered both winter and summer months. The level of infectivity varied considerably in the different lots of spores that were collected.

The mean results of 4 tests are given in Table II. Temperature of storage made but little difference and by 8 weeks there was less than 10 per cent of the original infectivity of fresh spores. Limited data indicated that there was no appreciable difference between 1 week and 2 weeks of storage.

Since the preparation of this paper the report by Schein (10) has ap-

TABLE I

EFFECT OF CARRIERS ADDED TO THE INOCULUM ON THE DEVELOPMENT OF RUST PUSTULES ON BEAN PLANTS

Carrier	Mean No. of pustules at 3 concentrations of carrier, mg. per 3 mg. spores			
	20	80	320	Mean
Talc	572	727	805	701
Montmorillonite (bentonite)	566	507	704	592
Silicon dioxide (Celite)	449	648	543	546
Pyrophyllite (Pyrax ABB)	357	449	698	501
Kaolinite (Cherokee clay)	554	497	418	489
Attapulgite (Attaclay)	314	459	473	415
Wood flour	443	277	192	304

TABLE II

INFECTIVITY OF UREDOSPORES STORED AT VARIOUS TEMPERATURES AND TIME PERIODS, EXPRESSED AS PERCENTAGE OF INFECTION AT ZERO TIME

Temp., °C.	Weeks in storage 2	4	8	16
22	25	23	5	0
18	38	20	8	0
15	39	20	4	0
10	33	17	4	0
5	36	20	6	1
0.5	39	18	8	1

peared. He found that the uredospores of bean rust when stored at temperatures of 23°, −13°, and −16° C. were dead after 1 to 5 months. But spores stored at −60° C. retained a residual viability of 40 per cent after 670 days, and infectivity was not impaired. If hydrated for 96 hours in a moist atmosphere before germination tests were made, germination levels above 70 per cent were obtained.

PRECISION OF FUNGICIDE TESTS

The ten fungicides listed above were compared in nine different tests on two replicate pots of plants for each concentration. The dose ratio was 5 and each fungicide was tested at 4 concentrations. The more effective chemicals were evaluated at 0.64, 3.2, 16, and 80 p.p.m. of formulated fungicides and the least effective at 16, 80, 400, and 2,000 p.p.m.; other fungicides were tested at intermediate concentrations. The ideal response sought was to cover the range from about 1 to 30 per cent infection or 99 to 70 per cent disease control based on the number of lesions on the control plants.

Weighting coefficients. There were available from the 9 experiments a total of 349 pairs of replicate pots of bean plants, i.e. plants treated at the same time with the same concentration of a given fungicide. The variances were calculated for the per cent disease on the paired replicates and the reciprocals or weight determined as described previously for early and late blights of tomato and snapdragon rusts (3, 4). A highly significant linear response was obtained for the logarithm of (weight × 10,000) plotted against the logarithm of (mean per cent disease × 10). The regression equation was $Y = 5.9022 - 1.4882X$ and standard error of estimate, $S_{y \cdot x}$, was 0.899. The standard error of the regression coefficient, S_b, was 0.06615 which gives a highly significant t value of 28.06 (8). This regression coefficient did not differ significantly from those obtained for the three above diseases. Hence the same weighting coefficients may be employed for all four diseases. At the ED95 level the logarithm of the (weight × 10,000) is 3.374. This may be compared with corresponding values of 3.265 for early blight, 3.850 for late blight, and 3.250 for snapdragon rust. Thus it may be concluded that within a test at the ED95 level, tests with bean rust under the above conditions give results about one-third as precise as late blight, but about 30 per cent more precise than with early blight or snapdragon rust.

Linearity of dosage-response curves. The dosage-response curves plotted on logarithmic-probability paper were tested for linearity by graphic examination of each of the nine replicate curves. For doubtful curves the data were converted to probits and the sum of the two outside points was compared to the sum of the two inside points. Significant curvature in one direction, i.e. at least 8 out of the 9 cases, was found for 5 of the fungicides. These results are shown graphically in Figure 2 A and B. The marked flattening of the nabam curve (Fig. 2 A) and of the maneb, ferbam, dichlone, and zineb curves (Fig. 2 B) indicates that even a very small amount of fungicide would give some control of these diseases under greenhouse conditions, while the opposite would be true with lime sulfur.

Fungicide × test interaction as error. The real criterion of differences in fungicides is the consistency of such differences in repeated tests. That is, the fungicide × test interaction should be used as the error term in making comparisons as has been emphasized previously (8, 9). With 6 of the fungicides, namely thiram, ferbam, maneb, zineb, dichlone, and captan finite responses were obtained at all 4 concentrations in all 9 tests. Furthermore the per cent control by each of the fungicides at a given concentration did not differ widely. Accordingly, an analysis of variance was run on the probit control at each level in order to demonstrate any differences in efficiency of comparing fungicides at different levels of control with the fungicide × test interaction used as the error term. The results are summarized in Table III A. It will be seen that no significant difference could be demonstrated at the two lower concentrations which gave mean per cent control respectively of 75.7 and 80.9. However, at the two higher concentrations with mean per cent control respectively of 88.7 and 97.9 a highly significant difference could be shown between the 6 fungicides. For comparison, data obtained earlier on tomato late blight (8) were calculated at four constant ED levels of 50, 80, 95, and 99 and are summarized in Table III B. Similar results were found. For both sets of data the variance for the error term, i.e. fungicide × test, remains more or less constant, while the variance for fungicides increases with the higher levels of control. Thus it can be demonstrated, as shown earlier (8, p. 120), that comparisons of fungicides can be made most efficiently at high levels of control such as the ED95.

An analysis of variance was made on the logarithm of the ED95 values obtained graphically for each of the 9 experiments on 8 of the fungicides. It was necessary to omit captan and zineb because of the low degree of control obtained in most tests. The variance for the fungicide × test error was 0.0850 with 56 degrees of freedom. This does not differ significantly from

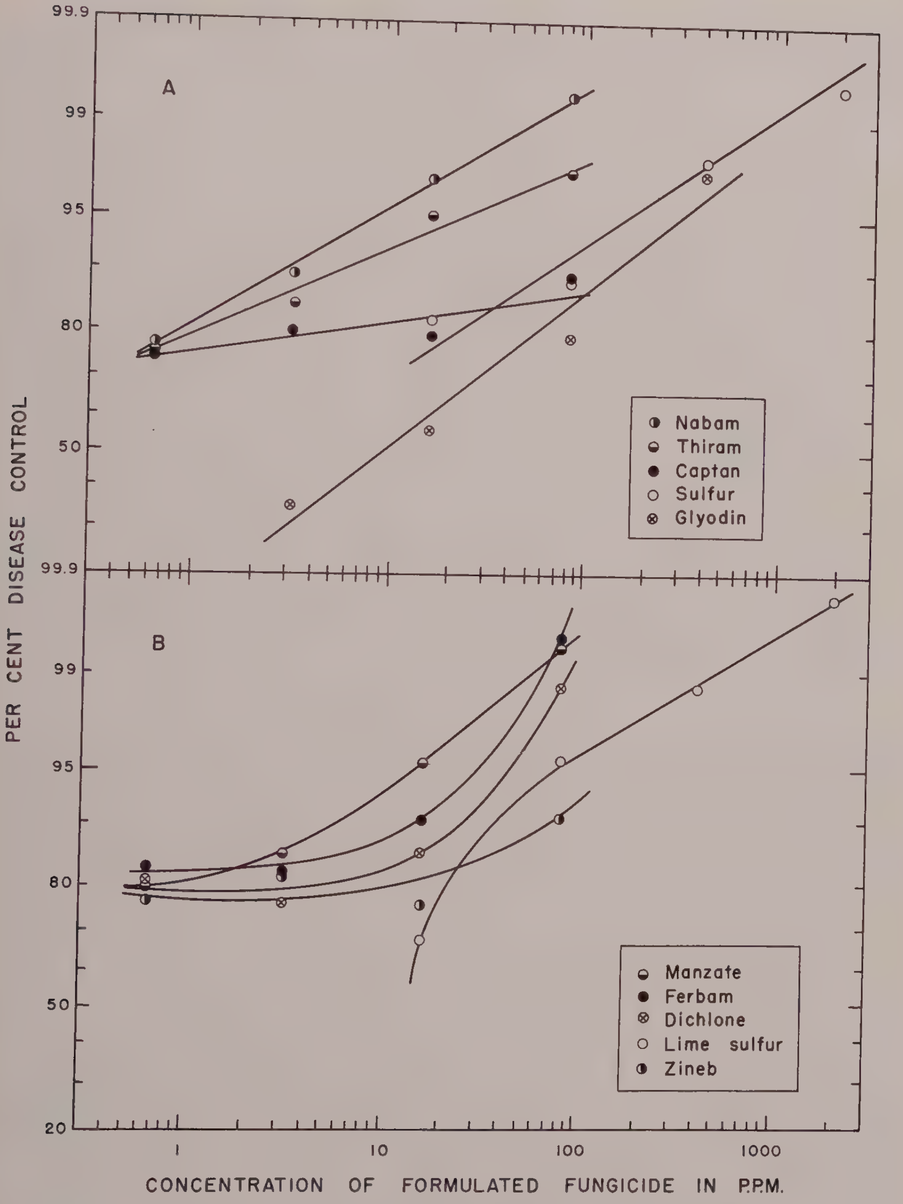

FIGURE 2. Dosage-response curves for the control of bean rust by various fungicides plotted on logarithmic-probability scale. Weighted mean of 9 tests at different times. Fungicides giving straight line responses in A, and significant curved responses in B.

TABLE III

COMPARISON OF FUNGICIDES AT DIFFERENT LEVELS OF CONTROL

A—Bean rust at constant concentration levels

Concn., p.p.m.	Mean % control	Fungicides		Fungicide × test		F value	Significance
		Var.	DF	Var.	DF		
0.64	75.7	0.187	5	0.193	40	<1.0	No
3.2	80.9	0.174	5	0.093	40	1.86	No
16	88.7	2.282	5	0.175	40	13.03	High
80	97.9	3.278	5	0.162	40	20.19	High

B—Tomato late blight. Data of 1943 (9) at constant ED levels

	Fungicides		Fungicide × test		F value	Significance
	Var.	DF	Var.	DF		
ED50	0.141	5	0.093	10	1.51	No
ED80	0.158	5	0.127	10	1.25	No
ED95	0.377	5	0.042	10	9.04	High
ED99	1.130	5	0.112	10	10.09	High

that of 0.1170 with 321 degrees of freedom for similar data on early and late blights of tomato (8). Thus the cumulative error variances for these earlier data may be used and differences of the order of 4.7-fold may be expected to show significance in 2 tests, while in 10 tests this would be reduced to a difference of some 67 per cent.

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RELATIONSHIP OF THE INSECTICIDAL AND THE FREE RADICAL ACTIVITIES OF DDT[1]

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SUMMARY

The isotope effect obtained by replacing the aliphatic *tertiary* hydrogen in DDT with deuterium is manifested by a retardation of chemical reactivity in free radical reactions such as dehydrochlorination, substitution, autoxidation, and chain transfer. This fact was used to test a hypothesis proposing that the chemical basis for the insecticidal efficacy of DDT is its potential for free radical formation. Quantitative insect toxicity, the timing of the appearance of poisoning symptoms in different species, and the effect of pretreating insects with *S*-(2-aminoethyl)isothiuronium bromide hydrobromide, a free radical antagonist, before exposure to DDT were measured. No evidence to support or invalidate the free radical proposal was obtained through these experiments designed to correlate the differential chemical and biological activity of DDT compounds with that of their deuterated analogues.

INTRODUCTION

The basic mechanism of action of one of the most successful organic insecticides, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) has eluded all investigations for over fifteen years. In addition to the numerous explorations of possible biochemical lesions that have been conducted seeking an explanation for its potent toxicity (13), proposals have been advanced suggesting that the insecticidal properties of this compound are dependent on precise spatial characteristics such as a definite structural topography (6), specific molecular dimensions (10), and a free rotability of its component parts (14). Such ideas have been difficult to subject to test through direct experimentation.

A recent hypothesis proposes that the insecticidal action of DDT is a result of its chemical reactivity, especially its capacity to participate in free radical reactions (9). The fact that DDT readily engages in free radical mechanisms such as chain transfer (11) supports the credibility of such a proposal. Replacement of the *tertiary* hydrogen atom of DDT and its analogues with deuterium (4) results in a significant, measurable isotope effect on chemical reactions involving cleavage of the carbon-hydrogen (C—H) or carbon-deuterium (C—D) bond such as in dehydrochlorination, substitution, autoxidation, or chain transfer (3). Thus, an excellent opportunity to assess the free radical-insect toxicity thesis seems to be offered.

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The present work was designed to determine if comparative toxicities to insects or the rate of appearance of poisoning symptoms produced by DDT and deuterated DDT (*d*-DDT) could be correlated with the decreased chemical rates of reactivity reflected by the isotope effect. Experiments were also performed to learn if the insecticidal action of DDT could be antagonized by a free-radical trapping agent.

MATERIALS AND METHODS

The house flies, *Musca domestica* L., employed in these tests were reared by standard procedures (12). Southern armyworm larvae, *Prodenia eridania* (Cram.), were raised on bean (*Phaseolus vulgaris* L. var. Tendergreen) plants and the mosquito larvae, *Aedes aegypti* (L.), were handled in prescribed fashion for the photomigration technique (2).

Topical applications of the chemicals were made on the mesonotum of five-day-old, adult female flies by means of a micrometer-driven syringe. The compounds were contained in one-microliter volumes of acetone, and the treated insects were held at room temperature. Mortality counts were made 18 hours after treatment.

Mosquitoes were exposed to *S*-(2-aminoethyl)isothiuronium bromide hydrobromide (AET) by placing the larvae in beakers containing the regular diet plus the protectant immediately following hatching. After 24 hours of contact, the larvae were transferred to fresh, deionized water. Measured quantities of DDT were added in minimal amounts of acetone, and an additional 24-hour holding period was observed before the photomigration tests were performed. Preliminary investigation indicated that AET was toxic at a concentration of 100 p.p.m., but no effects were noted at 10 p.p.m.

AET was incorporated in a skimmed milk-sugar solution and presented to recently emerged house fly adults. After five days, they were exposed to DDT by the bait feeding method (7).

Eggs of the southern armyworm were placed on bean foliage that had been dipped into water solutions of 1,000 p.p.m. AET and dried. Three days later the larvae were transferred to freshly-treated bean leaves to insure a constant supply of the AET salt. The Petri-dish biosassy (7) was used to compare the susceptibility of treated and untreated worms.

DISCUSSION

The deuterated analogues of DDT, 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethane (TDE) and 1,1,1-trichloro-2,2-bis(*p*-bromophenyl)ethane (DBrDT) were slightly more toxic to the house fly than the parent compounds (Table I). A similar difference between the paired DDT compounds was previously noted to be statistically significant (1). This differential susceptibility was not evident with compounds such as 1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane (methoxychlor) and 1,1-bis(*p*-methoxy-

TABLE I

COMPARATIVE TOXICITY OF *tertiary* CARBON TO HYDROGEN AND CARBON TO DEUTERIUM DDT AND DDT ANALOGUES FOR THE HOUSE FLY

Compound	LD50 (μg. topical/female fly)			
	DDT-susceptible (NAIDM strain)*		DDT-resistant (DDT-45 strain)**	
	C—H	C—D	C—H	C—D
1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT)	0.09	0.06	>20.0	>20.0†
1,1,1-Trichloro-2,2-bis(*p*-bromophenyl)ethane (DBrDT)	0.3	0.1		
1,1-Dichloro-2,2-bis(*p*-chlorophenyl)ethane (TDE)	0.5	0.4		
1,1,1-Trichloro-2,2-bis(*p*-methoxyphenyl)ethane (METHOXYCHLOR)	0.3	0.3		
1,1-Bis(*p*-methoxyphenyl)-2,2-dimethylpropane (DANP)	0.4	0.4		
1,1,1-Trichloro-2-(*o*-fluoro-*p*-chlorophenyl)-2-(*p*-chlorophenyl)ethane (*o*FDDT)	0.1	0.1	14.0	1.5
1,1,1-Trichloro-2-(*o*,*p*-dichlorophenyl)-2-(*p*-chlorophenyl)ethane (*o*ClDDT)	0.6	0.6	0.8	0.7
1,1,1-Trichloro-2-(*o*-bromo-*p*-chlorophenyl)-2-(*p*-chlorophenyl)ethane (*o*BrDDT)	0.4	0.4	2.0	1.7

* Standardized Natural Association of Insecticide and Disinfectant Manufacturers strain, never exposed to DDT.

** Obtained from Dr. A. S. Perry, U. S. Public Health Service, Savannah, Georgia. Resistant to DDT, Dilan, and Dieldrin.

† DDT at levels of 10, 20, and 40 μg. caused 0, 0, and 13 per cent kill respectively while *d*-DDT at these levels caused 16, 33, and 60 per cent kill.

phenyl)-2,2-dimethylpropane (DANP) in which the *para* halogens are replaced by methoxy groups.

A series of asymmetric DDT relatives in which *ortho* substituents were incorporated into one ring had been prepared and evaluated (8). A steric block such as this could possibly hinder the chemical phase of the intoxication process sufficiently to intensify the C—H, C—D isotope effect. No significant differences in toxicity to susceptible house flies were noted (Table I). The greatest variation in insecticidal properties was obtained with 1,1,1-trichloro-2-(*o*-fluoro-*p*-chlorophenyl)-2-(*p*-chlorophenyl)ethane against a strain of DDT resistant flies. This and the other less divergent results were invariably inversely correlated with the isotope effect. These differences may be the result of greater stability of the C—D bond compared with that of C—H in resisting detoxication involving attack at this point.

TABLE II

COMPARATIVE TOXICITY OF *tertiary* CARBON TO HYDROGEN AND CARBON TO DEUTERIUM DDT FOR THE HOUSE FLY IN RELATION TO TIME

Compound	Per cent mortality (0.07 μg. topical/female fly)					T50 (hours)
	Hours after treatment					
	2	4	8	16	32	
DDT (C—H)	0	4	16	50	94	18.0
DDT (C—D)	0	0	12	58	100	17.5
DDT (C—H)	0	0	8	54	100	17.8
DDT (C—D)	0	0	14	48	96	17.9

Since mortality is a terminal response, direct evidence concerning the free radical hypothesis was sought through an alternative parameter in which the isotope *rate* effect might prove decisive. The speed of action of the two related DDT compounds for the house fly, applied as topical microdrops, was measured, and the results are presented in Table II. No differences were found in T50 values or time required for 50 per cent mortality to be effected. Further, observations were made with mosquito larvae by means of the photomigration technique (2). The test, based on

TABLE III

COMPARATIVE TOXICITY OF *tertiary* CARBON TO HYDROGEN AND CARBON TO DEUTERIUM DDT FOR AEDES AEGYPTI LARVAE IN RELATION TO TIME

Materials tested	Per cent morbidity after exposure for 15–75 minutes					T50 (minutes)
	15	30	45	60	75	
DDT (C—H)	1	12	47	76		47
DDT (C—D)	1	16	51	76		46
Untreated control (1:30 p.m.)	1	1	4	4		
DDT (C—H)	1	1	16	47	72	62
DDT (C—D)	4	6	20	43	73	63
Untreated control (4:00 p.m.)	0	2	3	3	3	
DDT (C—H)	5	7	18	38	75	63
DDT (C—D)	6	7	30	59	74	56
Untreated control (5:10 p.m.)	4	4	6	8	9	
DDT (C—H)	1	21	58	78		44
DDT (C—D)	3	7	36	66		52
Untreated control (1:05 p.m.)	0	1	1	2		
DDT (C—H)	2	6	25	53	68	60
DDT (C—D)	2	14	34	58	69	57
Untreated control (2:15 p.m.)	3	5	5	5	6	
DDT (C—H)	0	1	14	27	45	79
DDT (C—D)	2	6	17	31	58	71
Untreated control (3:30 p.m.)	2	3	3	3	3	

TABLE IV

TOXICITY OF DDT TO INSECTS PRETREATED WITH *S*-(2-AMINOETHYL)ISOTHIURONIUM BROMIDE HYDROBROMIDE (AET)

Test insect	Method AET presentation	Method DDT presentation	DDT response	
			AET treated	Untreated
Mosquito larvae	48 Hours 1 p.p.m. soln. 48 Hours 10 p.p.m. soln.	Photomigration after 24-hr. exposure	67 70	73*
Southern armyworm larvae	Fed on bean leaves dipped in 1,000 p.p.m. until third instar	Treated bean leaves	50	40**
House fly adult	Fed milk with 1,000 p.p.m. for 5 days	Bait test	45 40	55†

* Average of 8 replicates (100 insects each) expressed as per cent morbidity.
** Average of 3 replicates (5 insects each) expressed as LD_{50} (p.p.m.).
† Average of 4 replicates (25 insects each) expressed as LD_{50} (p.p.m.).

negative phototropism, provided an objective and precise measurement of the time of onset of moribundity. The results of two sets of replicated experiments, performed serially at one-week intervals, disclosed no meaningful discrepancies between time-immobilization curves elicited by DDT and *d*-DDT or between the times required for 50 per cent inactivation of population (T_{50}) (Table III).

Sulfhydryl compounds have provided protection from radiation damage resulting from free radicals produced by ionizing irradiation of such structurally unrelated organisms as yeast (*Saccharomyces cerevisiae* Hansen) (15) and mice (5). By analogy, if DDT insect toxicity were indeed a reflection of free radical formation, then loading the insect with a chemical protectant prior to treatment with DDT might be expected to nullify, at least partially, the intoxication process. A series of experiments in which different insect species were pretreated with heavy doses of *S*-(2-aminoethyl)isothiuronium bromide hydrobromide, a recognized radical scavenger (5), before measuring the DDT response are summarized in Table IV. Mosquito larvae, *Aedes aegypti* (L.), grown in a continuous environment of 1 and 10 p.p.m. of AET for 48 hours, southern armyworm larvae reared to the third instar on bean leaves containing films of AET deposited by dipping in a 1,000 p.p.m. solution of AET, and adult house flies fed 1,000 and 5,000 p.p.m. AET in a diet of skimmed milk, all failed to show a differential susceptibility when tested with DDT in comparison with equivalent, non-AET-exposed controls. The results of these experiments suggest that either DDT produces no intoxicating free radicals or that the protectant was not present at the site of action in an active state at the time of intoxication.

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INITIAL PRODUCTS OF ACETATE UTILIZATION BY BEAN RUST UREDOSPORES[1]

Richard C. Staples

SUMMARY

Acetate was rapidly assimilated in small amounts by bean rust uredospores. Two per cent of the radioactivity from acetate-C^{14} was assimilated in 5 minutes. Radioactivity apparently entered the organic acid pool first, appearing in amino acids and sugars afterwards. Other fractions, especially protein, became radioactive very slowly. In the earliest periods studied, radiocarbon was found in glutamate and precursors of fatty acids such as malonate and an unknown compound (A) which has been tentatively identified as β-hydroxybutyrate. Shortly afterwards, C^{14} was found in intermediates common to the Krebs and glyoxylate cycles. Proportionately more C^{14} was converted to malate and sugars in later periods than was converted to malonate and unknown A. It was estimated from kinetic data that approximately 20 ± 10 per cent of the C^{14}-labeled isocitrate was metabolized via isocitric dehydrogenase, while the remainder was converted via isocitratase. The amount of C^{14} in amino acids was greater than in sugars, with glutamate and glutamine accounting for over 40 per cent of the C^{14} assimilated in 2 hours. Half of the C^{14} assimilated after 5 minutes from acetate-1-C^{14} was released as $C^{14}O_2$, while significant $C^{14}O_2$ release did not occur from acetate-2-C^{14} until after 2 hours. Evidence was obtained that malonate was initially decarboxylated to acetate before assimilation. Malonate was not assimilated as rapidly as acetate. Uredospores were found to be potentially very active in the synthesis of intermediate metabolites.

INTRODUCTION

Key enzymes of the glyoxylate and Krebs cycles have been demonstrated in uredospores (6, 7, 9, 27). While there has been some uncertainty concerning the importance of the Krebs cycle in uredospores (1, 15, 22), there is general agreement that it is potentially functional. The relative roles of the Krebs and glyoxylate cycles and their mutual relationship require clarification to provide a reasonable biochemical description of uredospores.

In general uredospores synthesize relatively small quantities of most materials. They do not effect a net synthesis of proteins and nucleic acids, and consumption of exogenous substrate is small (19). Chitin is synthesized in large amounts by uredospores during germination (16). Its most likely precursors, glutamine and glucose-6-phosphate (13), apparently are synthesized via the Krebs or glyoxylate cycles from endogenous fatty substrates.

No doubt this limited synthesis reflects the fact that uredospores do not grow. This makes it difficult to compare incorporation of many substrates

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in uredospores with their incorporation in rapidly growing tissues. Since, initially, acetate assimilation is significant, it was possible, in the short-term experiments reported here, to compare certain biosynthetic pathways in the uredospores with those of other tissues. Such a comparison might help to determine whether or not uredospores differ significantly from tissues capable of sustained growth. A preliminary account of this work has been published elsewhere (18).

MATERIALS AND METHODS

Uredospores of *Uromyces phaseoli* (Pers.) Wint. were obtained from greenhouse grown bean plants (*Phaseolus vulgaris* L. var. Pinto). Spores were stored at 5° C. for not more than 2 weeks after harvest.

Sodium acetate-1-C^{14} and sodium acetate-2-C^{14} employed in these studies each had a specific activity of 2 millicuries per millimole, while sodium malonate-2-C^{14} had a specific activity of 0.915 millicurie per millimole. The specific activity of sodium bicarbonate-C^{14} was 1 millicurie per millimole.

Incubation of spores with various C^{14}-labeled substrates was carried out in 1-liter flasks on a shaker. Usually, 1 g. of spores was suspended in 100 ml. of 0.01 per cent Tween 20 in water containing the substrate. After the desired incubation period, 300 ml. of boiling absolute ethanol was added and the spores were extracted as described previously (19). The extracts were combined, fractionated with ion exchange resins, and the individual fractions analyzed quantitatively as before (19). Sugars were determined by automatic chromatographic analysis (21).

When the technique of isotopic competition was employed usually 1 g. of uredospores was incubated with the nonradioactive compound and after 30 minutes 10 μc. sodium acetate-1-C^{14} was added. After termination of the experiment the spores were extracted as usual.

Paper chromatography and autoradiography of amino acids, organic acids, and sugars were carried out as described previously (20, 23). Radioactivity of the various fractions, column effluents, carbon dioxide, and hydrolyzates was measured on dry samples in aluminum planchets using a thin-end window gas flow counter.

After isolation, malic acid was degraded according to the procedure of Bradbeer, Ranson, and Stiller (4). Amino acids were degraded according to Aronoff (2, p. 185).

RESULTS

IDENTIFICATION OF COMPOUNDS

Citrate and malonate (17), pyrrolidone carboxylate, succinate, malate, and fumarate have been isolated previously from bean rust uredospores (22). Aconitate and trehalose were identified by column and paper chromatographic (10, 14, 23, 25) techniques. The spores contained 136 ± 3 μg.

of trehalose per mg. protein N. Two anthrone-positive compounds with high Rf values in common solvents remain unidentified. Amino acids were identified by 2-dimensional chromatography on filter paper (19).

An unidentified acid was also detected on autoradiograms. It was present only in trace amounts and could not be titrated by the usual procedure for determining organic acids. Its position on the autoradiogram suggested that it might be β-hydroxybutyrate. Chromatography with authentic β-hydroxybutyrate confirmed this possibility since the two compounds did not separate in the solvent systems described by Palmer (14) and Stark, Goodban, and Owens (23).

INTERCONVERSION OF ORGANIC ACID INTERMEDIATES

The formation of organic acids from glyoxylate and α-ketoglutarate was studied, and the results are presented in Table I. Regardless of the route, carbon evidently tends to accumulate in succinate, while malate and fumarate are rapidly turned over. Both glyoxylate and α-ketoglutarate were converted to succinate with a reduction in its specific activity. Some malate was formed from α-ketoglutarate, but only a slight increase was detectable from glyoxylate.

TABLE I

INTERCONVERSION OF ORGANIC ACID INTERMEDIATES AS DETERMINED BY ISOTOPIC COMPETITION. ONE GRAM OF BEAN RUST UREDOSPORES WAS INCUBATED WITH GLYOXYLATE OR α-KETOGLUTARATE FOR 30 MINUTES, THEN FOR 10 SECONDS WITH 10 μC. SODIUM ACETATE-1-C^{14}

Organic acid assayed	Uredospores incubated 30 minutes with					
	Water		Glyoxylate		α-Ketoglutarate	
	μMoles	C.p.m./ μmole	μMoles	C.p.m./ μmole	μMoles	C.p.m./ μmole
Citrate	4,560	0	4,061	Trace	4,900	0
Fumarate	Trace	0	—	0	174	0
Malate	134	0	150	0	268	0
Malonate	1,232	Trace	1,200	0	1,326	Trace
Succinate	230	8	690	0	2,124	1

INITIAL PRODUCTS OF ACETATE UTILIZATION

General distribution of radioactivity.[2] Data in Figure 1 A and B show the distribution of C^{14} from acetate-1-C^{14} and acetate-2-C^{14} in various fractions of uredospores. In general, assimilation of acetate was slow, amounting only to 2 per cent of that originally present in the medium after 5 min-

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[2] The time for alcohol penetration and killing is unknown. Intervals shorter than 1 to 5 minutes must be considered relative and only as a way of designating when alcohol was added to stop the action.

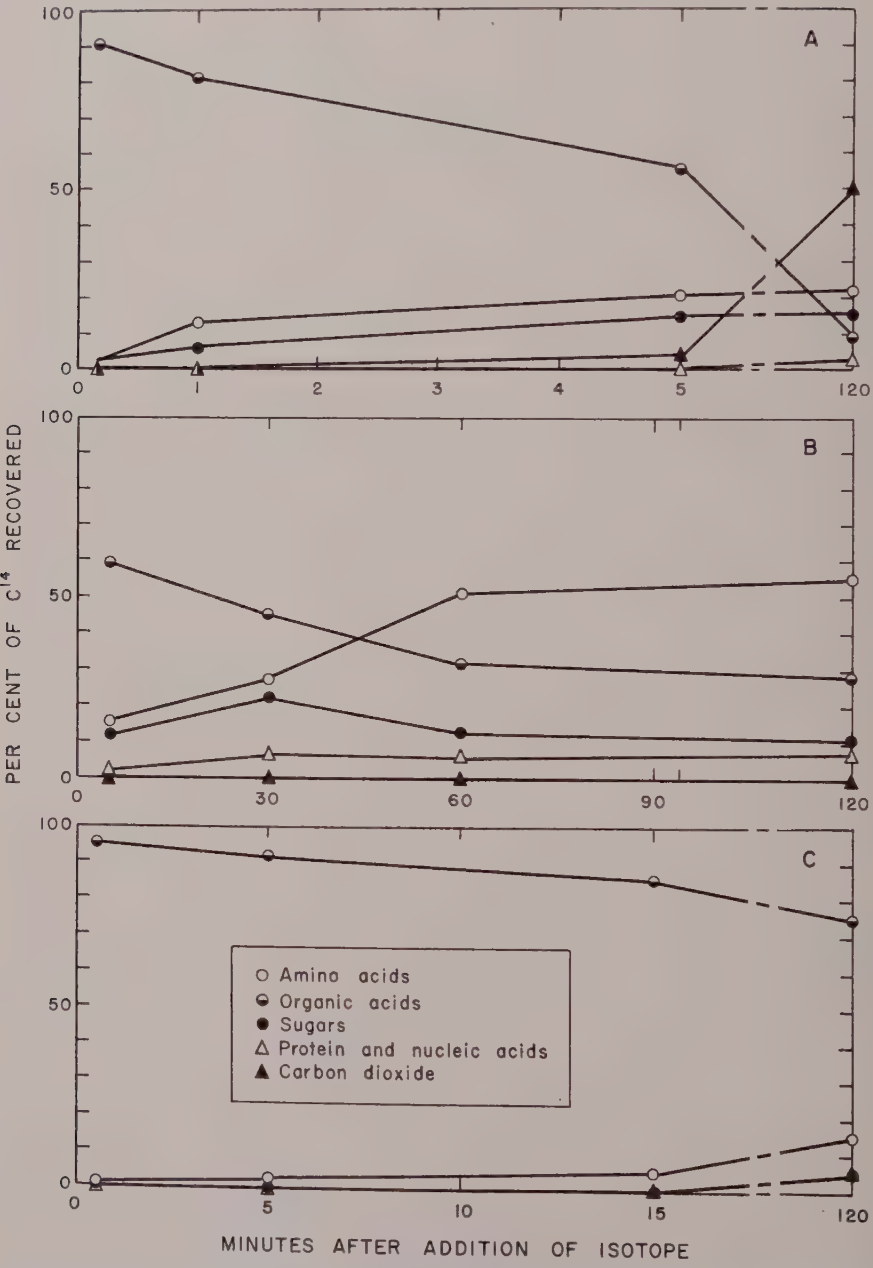

FIGURE 1. Distribution of C^{14} in various fractions extracted from bean rust uredospores after their exposure to 10 μc. of: A. Sodium acetate-1-C^{14}; B. Sodium acetate-2-C^{14}; C. Sodium malonate-2-C^{14}.

utes. Malonate incorporation was even slower and significant amounts of C^{14} were not detected until after 15 minutes (Fig. 1 C).

The whole acetate molecule was incorporated into the acids, since the incorporation patterns for methyl- and carboxyl-labeled acetates were similar. For example, the negative slopes of the organic acid curves indicate that C^{14} was initially incorporated into the organic acid fractions. They also show that sugars were labeled within 1 minute, while significant label did not appear in protein until after 2 hours. Long-term studies make it clear that eventually the sugars become more strongly labeled than amino acids, because after 5 hours, 40 per cent of the assimilated C^{14} was in sugars.

Important differences in utilization of methyl- and carboxyl-labeled acetates can be seen when comparing the release of CO_2 by uredospores incubated with these substrates. When carboxyl-labeled acetate was employed, $C^{14}O_2$ appeared within 5 minutes, and half of the C^{14} assimilated was evolved as $C^{14}O_2$ after 2 hours. When methyl-labeled acetate was employed, $C^{14}O_2$ evolution was delayed beyond 2 hours. This conservation of C^{14} undoubtedly explains the stronger labeling of the protein and nucleic acid fractions compared to uredospores incubated with acetate-1-C^{14}.

Early products of acetate assimilation. The distribution of radioactivity from acetate-1-C^{14} into various components of uredospores is shown in Table II. Succinate was the first Krebs cycle acid to become radioactive, but malonate, glutamate, and unknowns A and B were labeled earlier. Succinate and aspartate became radioactive within 10 seconds, and malate within 30 seconds. Citrate and aconitate were labeled within 1 minute.

TABLE II

Distribution of C^{14} in Various Components of Bean Rust Uredospores after Incubation with 10 μC. Sodium Acetate-1-C^{14} (6.2×10^6 C.P.M.) at Various Time Intervals

Product	Per cent total c.p.m. assimilated in 3 to 300 sec.				
	3	10	30	60	300
Aconitate	0	0	0	1	1
Aspartate	0	10	4	4	Trace
Citrate	0	0	0	15	15
Fumarate	0	0	0	4	9
Glutamate	14	18	6	4	3
Malate	0	0	4	28	30
Malonate	3	19	11	8	Trace
Succinate	0	22	35	15	11
Sugars	0	3	4	6	15
Unknown A	46	30	30	14	15
Unknown B	34	0	4	0	0
Total c.p.m.	3,800	6,000	7,100	19,700	33,300
Total c.p.m. in CO_2	—	—	Trace	200	1,500

TABLE III

Specific Activities of Early Products of Acetate Utilization after Incubation with 10 μC. Sodium Acetate-1-C^{14} for Various Intervals. Data Are Given in C.P.M./μMole

Product	3 Sec.	10 Sec.	30 Sec.	60 Sec.	300 Sec.
Citrate	0	0	0	1.4	1.8
Fumarate	0	0	0	0.1	30
Malate	0	—	0.2	19	22
Malonate	0.5	0.6	0.4	3	Trace
Succinate	0	0.7	5	24	59

The specific activities of some of these intermediates are given in Table III. The activity values are directly related to endogenous content of the acids in the tissue (cf. Table I), except for citrate. A similar observation was also made when uredospores were incubated with $NaC^{14}O_3$ (22).

Isotope competition experiments. When nonradioactive glyoxylate was employed with acetate-1-C^{14} for 10 seconds (Table IV), the incorporation of radioactivity into succinate and malonate was suppressed, but citrate and aconitate became radioactive.

INTERNAL LABELING

Malate. Distribution of C^{14} in malate following its isolation and degradation is given in Table V. Approximately 45 per cent of the radioactivity was in C_1, while 55 per cent was in C_4. The distribution changed only to 40:60 when acetate assimilation proceeded for 2 hours. Radioactivity was not detected in the center carbons. The presence of nonradioactive glyoxylate significantly reduced C^{14} in C_1 as would be expected if most of the malate were formed via the glyoxylate pathway.

TABLE IV

Distribution of C^{14} in Organic Acids from Bean Rust Uredospores after 30 Minutes Incubation with 10 Mg. Sodium Glyoxylate Followed by 10 Seconds with 10 μC. Sodium Acetate-1-C^{14}

Organic acid formed	Per cent of total c.p.m. assimilated into organic acids after incubation for 30 minutes with	
	Water	Glyoxylate
Aconitate	0	29
Citrate	0	13
Fumarate	0	0
Malate	0	0
Malonate	19	20
Succinate	22	0
Unknown A	30	30
Total c.p.m.	6,000	1,000

TABLE V

DISTRIBUTION OF C^{14} WITHIN MALATE EXTRACTED FROM BEAN RUST UREDOSPORES EXPOSED TO 10 μC. SODIUM ACETATE-1-C^{14} AFTER A PRIOR 30-MINUTE EXPOSURE TO 10 MG. SODIUM GLYOXYLATE

Carbon	Per cent total C^{14} in malate after various periods of exposure to isotope		
	5 Minutes and water	2 Hours and water	5 Minutes and glyoxylate
C_1	45	41	6
$C_{2,3}$	0	0	0
C_4	55	59	94
Total c.p.m.	10,000	47,000	2,000

Krebs cycle amino acids. After incubation of uredospores for 2 hours with sodium acetate-2-C^{14}, aspartate, alanine, and glutamate were isolated and decarboxylated with ninhydrin. The results appear in Table VI. Radioactivity was not detected in the C_1 of the amino acids. These patterns would be expected if most of the malate was formed via the glyoxylate pathway.

UTILIZATION OF MALONATE-2-C^{14}

Distribution of C^{14}. Malonate-2-C^{14} was converted slowly to amino acids, sugars, and CO_2 (Fig. 1 C). The conversion of malonate-2-C^{14} to intermediates and carbon dioxide quantitatively resembles the conversion of acetate-2-C^{14} to these substances. For example, amino acids acquired more C^{14} than sugars did. However, nearly 15 minutes elapsed before significant amounts of C^{14} were found in amino acids. Less data are available for malonate-1-C^{14}, but the amount of C^{14} in CO_2 equaled that in amino acids.

Early products of malonate assimilation. Table VII depicts the distribution of radioactivity among various intermediates extracted from uredospores after assimilation of malonate-2-C^{14}. The activity was mainly in unidentified fatty acids, but could be detected in glutamate and organic acids, especially succinate.

TABLE VI

NINHYDRIN DEGRADATION OF SEVERAL AMINO ACIDS ISOLATED FROM BEAN RUST AFTER EXPOSURE FOR 2 HOURS WITH 5 μC. SODIUM ACETATE-2-C^{14}

Amino acid	C.p.m. in	
	CO_2	Residue
Alanine	0	230
Aspartate	0	650
Glutamate	0	1,260

TABLE VII

ASSIMILATION OF MALONATE-2-C^{14} BY BEAN RUST UREDOSPORES AFTER INCUBATION FOR VARIOUS PERIODS WITH 50 μC. OF THE ISOTOPE

Product	Per cent total C^{14} assimilated after		
	1 Minute	5 Minutes	15 Minutes
Aconitate	6	6	6
Aspartate	0	0	Trace
Citrate	0	0	3
Fumarate	1	1	1
Glutamate	1	1	22
Glutamine	0	0	Trace
Malate	1	1	4
Succinate	6	5	10
Unknown	53	39	39
Total c.p.m.	11,000	14,000	35,000

Carbon dioxide evolution. A study of carbon dioxide evolution from malonate was made in order to clarify the pathway of malonate degradation. The evolution of carbon dioxide from malonate-2-C^{14} was delayed until 15 minutes when the CO_2 had an activity of only 50 c.p.m. Radiocarbon from malonate-1-C^{14} passed into CO_2 much more rapidly and appeared by 1 minute. After 15 minutes the activity of the CO_2 had reached 1.1×10^6 c.p.m. Since the total activity fixed by the spores was 10^6 c.p.m., it is clear that the same activity appeared in the spores as was evolved in CO_2. This would be expected if malonate were being converted to acetate by an initial decarboxylation.

Malonate metabolism by cell-free breis. If uredospores converted malonate to acetyl-coenzyme A (26), the addition of hydroxylamine to a cell-free brei should reveal malonyl and acetyl hydroxamates (28). Paper chromatography of the deproteinized brei revealed malonyl dihydroxamate within a short time after addition of malonate-2-C^{14} or $NaHC^{14}O_3$. Acetyl hydroxamate-C^{14} was demonstrated only by incubation of the brei with malonate-2-C^{14} for an hour before addition of hydroxylamine.

DISCUSSION

Enzymes of the glyoxylate and Krebs cycles have been demonstrated in uredospores (6, 7, 9, 27), and evidence has now been obtained that both cycles operate. The important end-products of this synthetic activity are sugars and glutamine which apparently are utilized mainly for chitin synthesis (13), the major product of synthesis by germinating uredospores (16).

Several synthetic pathways exist for some compounds. Feeding experiments showed that malate-C^{14} was synthesized via both malate synthetase and malic dehydrogenase. However, nonradioactive glyoxylate strongly reduced labeling in C_1 of malate so that the synthesis of malate was largely via malate synthetase. The distribution of C^{14} in malate isolated from uredospores was quite similar to that isolated from sunflower cotyledons (3). In castor bean tissue where Krebs cycle activity was not operative, C^{14} was equally distributed between C_1 and C_4 (5).

By relating the C^{14} found in various compounds known to be produced predominately by either the glyoxylate (sugar) or Krebs cycles (CO_2), it has been possible to estimate that approximately 20 ± 10 per cent of the C^{14}-labeled isocitrate was metabolized via isocitric dehydrogenase, while the remainder was converted via isocitratase. The ratio of c.p.m. in CO_2 to c.p.m. in sugar was 0.2 at 1 minute and 0.3 at 5 minutes. This would indicate that between 20 and 30 per cent of the isocitrate was split via isocitric dehydrogenase, the remainder being converted via isocitratase. A ratio of c.p.m. in glutamate to c.p.m. in sugar is also 0.2. The average of these estimations thus approaches 20 ± 10 per cent. This cannot be taken as a rigorous figure, but it does indicate that most of the acetate must be metabolized via the glyoxylate pathway. Furthermore, much of the C^{14} entering the Krebs cycle apparently accumulates as glutamate, so that the cycling of C^{14} away from α-ketoglutaric dehydrogenase must be quite small. The extremely low evolution of $C^{14}O_2$ from acetate-2-C^{14} is in agreement with these conclusions, as is the internal labeling of the amino acids (Table VI) and malate (Table V).

Nonradioactive glyoxylate eliminated radioactivity in succinate (Table III), which suggests that succinate was derived primarily from malate via malic synthetase rather than from α-ketoglutarate. To suggest that the observed primary formation of succinate could be the result of condensation of 2 moles of acetyl CoA as it has been for nongrowing *Escherichia coli* (8) would be tempting, but several factors dispel such an effort. (a) Glutamate was formed before succinate. (b) When glyoxylate was added to the spores an earlier detection of C^{14} in citrate and aconitate was possible, and short-term results may therefore depend upon large differences in pool sizes which occur in nongrowing tissues. (c) Partial anaerobiosis has been suggested as a cause for the early detection of succinate (12), and such conditions probably did exist during the experiment. (d) It has been pointed out that normal operation of the glyoxylate pathway would provide a sufficient mechanism for formation of succinate before citrate (11). Kornberg has critically discussed other evidence concerning the formation of succinate from acetate (8, 12).

Radioactivity was first detected in malonate and an unknown acid tentatively identified as β-hydroxybutyrate. Malonate was probably formed by carboxylation of acetyl coenzyme A as in other tissues (24, 26), since the data, including work with cell-free breis, can best be interpreted by using this concept. Malonate is also synthesized from $C^{14}O_2$ (22).

Acetate was rapidly assimilated by uredospores in trace amounts. This early assimilation slows down, nearly stops by 10 hours, and C^{14} is lost by 24 hours (19). It would seem that uredospores are potentially very active in the synthesis of small molecules. Their failure to grow and to synthesize protein and nucleic acids seems to be the major factor causing the truncated patterns of synthesis actually obtained.

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